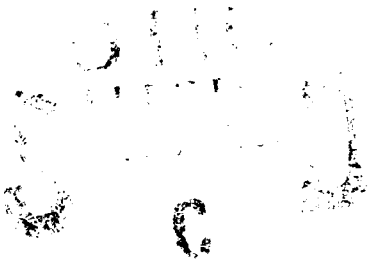


CONTRACT NO: DAMD17-88-C-8035

TITLE: NOVEL APPROACHES FOR TARGETING ANTIVIRAL AGENTSIN THE TREATMENT OF ARENA-, BUNYA-, FLAVI-, AND RETROVIRAL INFECTIONS

PRINCIPAL INVESTIGATORS: J. David Gangemi, Ph.D.
Abdul Ghaffar, Ph.D.
Eugene P. Mayer, Ph.D.

CONTRACTING ORGANIZATION: University of South Carolina
School of Medicine
Columbia, South Carolina 29208

REPORT DATE: June, 1990

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: CLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of South Carolina School of Medicine; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Columbia, SC 29208	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-88-C-8035
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62787A	3M1 62787A871	AH	373

11. TITLE (Include Security Classification)
Novel Approaches for Targeting Antiviral Agents in the Treatment of Arena-, Bunya-, Flavi-, and Retroviral Infections

12. PERSONAL AUTHOR(S)
J. David Gangemi, Ph.D., Abdul Ghaffar, Ph.D., and Eugene P. Mayer, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 12/13/88 TO 6/1/90	1990 June	85

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Viruses, Neoglycoproteins, BD, RAI, Liposomes, Antiviral, Immunostimulated, Lab Animals
06	13		
06	15		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Administration of multiple doses of Poly I:C-LC, Riker-3M or CL 246878 every other day for ten days did not prolong activation of immune functions. In some cases this treatment actually suppressed immune functions. Nevertheless, administration of multiple doses of these drugs had no adverse effect on resistance to Banzi virus infection. Encapsulation of Poly I:C in liposomes resulted in a formulation which was more effective than free drug in augmenting immune functions and resistance to virus challenge. Poly I:C conjugated to dextran was superior to free Poly I:C-LC in inducing interferon and stimulating the reticuloendothelial system. In addition, Poly I:C-dextran conjugates did not have the apparent toxic effects on the reticuloendothelial system that were observed with comparable doses of Poly I:C-LC. Type 2 dengue virus (strain 16681) infects squirrel monkeys as evidenced by both viremia and induction of anti-dengue antibodies. This virus model provides an opportunity to evaluate the therapeutic potential of immunomodulating and antiviral agents in non-human primates infected with an arbovirus.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Virginia M. Miller	(301) 663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWARD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

__X__ Where copyrighted material is quoted, permission has been obtained to use such material.

__X__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__X__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

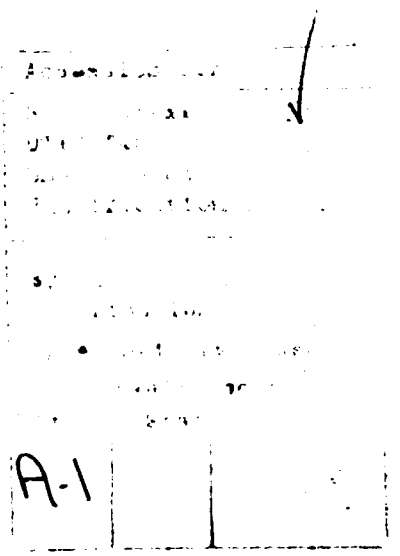
__X__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resource, National Research Council (NIH Publication No. 86-23, Revised 1985).

__X__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

6/25/90

PI - Signature Date



SUMMARY

Administration of multiple doses of Poly I:C-LC, Riker-3M compound or CL 246878 every other day for ten days did not prolong activation of immune functions. In some cases this treatment actually suppressed immune functions. Nevertheless, administration of multiple doses of these drugs had no adverse effect on resistance to Banzi virus infection. Encapsulation of Poly I:C in liposomes resulted in a formulation which was more effective than free drug in augmenting immune functions and resistance to virus challenge. Poly I:C conjugated to dextran was superior to free Poly I:C-LC in inducing interferon and stimulating the reticuloendothelial system. In addition, Poly I:C-dextran conjugates did not have the apparent toxic effects on the reticuloendothelial system that were observed with comparable doses of Poly I:C-LC. Type 2 dengue virus (strain 16681) infects squirrel monkeys as evidenced by both viremia and induction of anti-dengue antibodies. This virus model provides an opportunity to evaluate the therapeutic potential of immunomodulating and antiviral agents in non human primates infected with an arbovirus.

TABLE OF CONTENTS

LIST OF APPENDICES

Tables

Figures

PROBLEM UNDER INVESTIGATION

A. BACKGROUND AND HYPOTHESIS

A number of clinically proven antivirals, such as amantadine, iododeoxyuridine, adenine arabinoside, acycloguanosine, ribavirin, and azidodeoxythymidine are currently in use. In addition, several potentially useful antivirals (*e.g.* S-HMPA, selenazole, WIN 5177, arildone, phosphonoformic acid) are currently under investigation. There are, however, toxicity problems associated with the use of antivirals at doses required to provide sustained drug levels in virus infected organs. Because of the many similarities in synthetic "machinery" and pathways used by both viruses and mammalian cells, these toxicity problems may be difficult to overcome. Furthermore, in some virus infections even the presence of a highly active antiviral in the infected target organ may not be sufficient to prevent or limit disease. Thus, a delivery system which can provide slow release of an antiviral, alone or in combination with other effector substances (*e.g.*, immunostimulants, antibody, interferon), at the site of primary virus replication may be highly desirable. We believe, that with the recent technological advances in the development of carrier vehicles designed to protect nontarget environments from the drugs they carry, and facilitate slow release of one or more drugs at sites at which they are needed, many of the problems currently associated with antiviral chemotherapy can be minimized.

A.1 Rationale and theoretical considerations for the use of targeted drug carriers

Successful drug use in medicine is often jeopardized by the failure of drugs that are otherwise active *in vitro* to act as efficiently *in vivo*. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In addition, drugs may be prematurely excreted or inactivated; thus, an effective therapeutic drug concentration may not be obtained at the site of infection. While this problem can be overcome by increasing dosage, this often results in toxicity. For example, a number of antiviral agents (ribavirin, adenine arabinoside, phosphonoformic acid, azidodeoxythymidine) have been developed which are highly effective *in vitro* in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression (1,2) which may affect the outcome of treatment since recovery from most viral infections involves the cooperation of host immune responses. There is now growing optimism that these problems may be resolved or minimized with the use of carrier vehicles such as liposomes, monoclonal antibodies or conjugated proteins. These vehicles not only protect the immune system from the drugs they carry but also deliver and facilitate drug release at site(s) at which they are needed.

A prerequisite for the successful use of antiviral carriers is that they recognize the target site and release drug in an active form. Thus, the antiviral must be linked to a carrier in a stable fashion so that it remains attached throughout its journey from the site of inoculation to the target. For this to occur, the drug-carrier linkage must be resistant to the various enzymatic and physiochemical conditions prevailing in the bloodstream and extracellular fluids. This stability also implies that the drug remains inert as long as it is associated with its carrier so that it will be inactive prior to reaching the target area. A corollary of this requirement is that there be a mechanism by which the drug will be activated after reaching its destination. Three possibilities exist for the activation of carrier bound drugs: 1) endocytosis and release in acidic endosomes; 2) extracellular activation at cell surfaces by plasma membrane associated enzymes; and 3) intracytoplasmic penetration via transmembrane passage.

During the past decade, the use of carriers for the selective targeting of antitumor drugs has been advocated with increasing frequency. This has led to numerous reports on the association of drugs or toxins such as anthracyclines, methotrexate, bleomycin, chlorambicin, cytosine arabinoside (ara-C), and ricin with carriers such as DNA (3,4), liposomes (5,6), monoclonal antibodies (7-9), hormones (10,11), red blood cell ghosts (12), neoglycoproteins (13) and other proteins (14,15). Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment where free drug is released when the bond between drug and carrier is hydrolyzed by lysosomal enzymes (16). In contrast, some liposomes deliver their contents directly into the cytoplasm following fusion with the target cell membrane (17). This mode of delivery is useful for antivirals which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which drug contact with lysosomes can be bypassed. In addition, liposomes are useful in the delivery of poorly soluble agents such as arsenicals in the treatment of leishmaniasis (18) and lipophilic drugs such as MTP-PE in the treatment of herpesvirus infections (19,20). Moreover, they provide a means to deliver drugs that cannot be transported across cell membranes.

A.1.1 Liposomes

Four basic liposome designs: 1) multilamellar; 2) small unilamellar; 3) large unilamellar; and 4) vesicles produced by reverse phase evaporation, have been used as carrier systems for enhancing the pharmacological activity of drugs and the incorporation of macromolecules into cells. The most commonly used methods for the preparation of liposomes are the multilamellar vesicles originally described by Bangham *et al.* (21), and the small unilamellar vesicles initially described by Papahadjopoulos and Miller (22). The main drawbacks of these "classical" liposome preparations are the wide heterogeneity in size distribution and number of lamellae, the relatively low trapping efficiency in the aqueous space and/or their inability to encapsulate large macromolecules due to their small

internal volume (23). In contrast, newer methods in the preparation of large unilamellar and reverse phase evaporation liposomes have resulted in carriers with larger internal volumes and better encapsulation properties. For example, a reverse evaporation process has been used to produce unilamellar vesicles capable of encapsulating substantial amounts of ara-C (24). These liposomes were composed of phosphatidylserine and cholesterol and were stable in serum. Furthermore, their distribution to lung, liver and spleen following intravenous inoculation could be controlled, in part, by altering one or more physical features (*e.g.*, size, membrane fluidity and charge,) or by embedding of glycoconjugates, proteins etc.

Comparison of pulmonary retention of liposomes of differing size, surface charge, and composition following iv injection revealed that large multilamellar or reverse phase evaporated liposomes arrested in the lung more efficiently than small unilamellar liposomes of identical lipid composition. In addition, multilamellar and reverse phase evaporation vesicles containing negatively charged amphophiles arrested in the lung more efficiently than neutral or positively charged structures which accumulated in the liver and spleen (25). Reverse phase evaporation carriers were superior to multilamellar carriers with respect to their loading capacity, however, the latter provided a more prolonged drug release.

Liposomes containing glycolipid have a preferential affinity for hepatocytes due to a galactose binding membrane lectin (26). Using liposomes which varied in size and galactosyl lipid content (galactocerebroside), Rahman *et al.* were able to show more selective targeting of small unilamellar vesicles to hepatocytes (27). Similarly, large liposomes containing galactocerebroside produced a substantial enhancement of liposome-encapsulated primaquine activity against the schizont stage of malarial parasite which is known to reside in hepatocytes (18).

In many instances, liposomes are avidly taken up by tissue associated macrophages which are present in most organs. Selective uptake of these vehicles by macrophages can be quite useful and can be exploited in some infectious diseases; thus, Alving *et al.* (18) used liposome-encapsulated antimonial drugs to treat leishmaniasis, a parasitic infection in which the macrophage is the primary target of infection. Encapsulated drug preferentially accumulated in these cells and killed the parasite with minimal toxicity for nontarget cells.

In addition to the examples cited above, liposomes have been used as carriers to enhance the immunogenicity of a variety of viral proteins including hepatitis B surface antigen, vesicular stomatitis virus glycoproteins, and adenovirus capsid proteins. Encapsulation of these proteins and inoculation into animals resulted in an increased production of serum neutralizing antibodies (28-30). In at least one study, in which purified major surface glycoproteins of VSV were encapsulated into liposomes, both specific humoral and cell mediated immunity were enhanced (28).

Recently, attempts to load antivirals into freeze-dried liposomes has resulted in improved encapsulation with minimal drug-leakage upon storage (personal communication, Dr. Peter van Hoogevest). This is particularly significant since it should now be possible to more easily load a variety of antivirals and/or immunostimulants into this type of carrier. We have recently encapsulated both ribavirin and muramyl tripeptide phosphatidylethanolamine (MTP-PE) into freeze-dried liposomes and have demonstrated their usefulness in the treatment of viral diseases. Thus, in the future, liposomes may become a more widely used vehicle for delivery of drugs in antiviral chemotherapy.

A.1.1.1 Liposome targeting of antivirals

One of the early applications of liposomes as a delivery vehicle for antivirals was made in the treatment of herpes keratitis with iododeoxyuridine (31). In this study, liposome encapsulated iododeoxyuridine was shown to be more effective than free drug in the treatment of both acute and chronic keratitis. In another study Kende *et al.*(32) showed a five-fold increase in liver concentrations of ribavirin following liposome encapsulation. In addition, encapsulated drug was more effective than free drug in the treatment of mice infected with the hepatotropic Rift Valley fever virus. More recently, we have demonstrated a five-fold increase in pulmonary concentrations of ribavirin when drug was encapsulated in liposomes and administered intravenously (20). Liposome-encapsulated ribavirin was more effective than free drug in protecting mice against lethal challenge with influenza virus. Thus, tissue targeting of antivirals with liposomes substantially increases their therapeutic index.

A.1.1.2 Liposome targeting of immunostimulants

Liposome encapsulated immunomodulators such as macrophage activating factor (MAF) and muramyl dipeptide (MDP), or its lipophilic analogue (MTP-PE), have been used to stimulate pulmonary macrophage activity and enhance host resistance to lung metastasis (26,33,34) and herpesvirus infection (19,20). Koff *et al.* (19) have shown that liposome-encapsulated MTP-PE was more effective than free MTP-PE in preventing death in a murine model of HSV-2 hepatitis. Similarly, we have demonstrated that liposome encapsulated MTP-PE was more effective that free drug in a murine model of HSV-1 pneumonitis (20). Protection observed with liposome encapsulated drug appeared, in part, to be due to enhanced drug localization in the liver and lungs of infected mice. In another study, it was shown that liposome-encapsulated MTP-PE was more effective than free drug in the healing of genital lesions induced by HSV-2 infection of guinea pigs. Therefore, liposome encapsulation of immunostimulants can be used to enhanced localization of drugs in various tissues, thus providing an effective stimulus for the activation of local macrophages.

A.1.2 Drug/Glycoprotein conjugates

Drug conjugated glycoproteins or neoglycoproteins (proteins such as serum albumin to which sugars have been attached) are promising targeting vehicles because they are easily prepared, biodegradable, and nontoxic. Fiume *et al.* (35-37) have shown that drugs can be stably conjugated to asialofetuin or galactosyl-terminating serum albumin and bind to lectin receptors on hepatocytes (38,39). Selective uptake of these intravenously administered drug conjugates resulted from the binding of carrier to galactosyl binding sites on hepatocyte membranes (35). Quite remarkably, drugs conjugated to serum albumin were selectively cleared by neighboring liver sinusoidal endothelial and Kupffer cells (37). Once inside these cells, the drug-conjugates entered lysosomes where hydrolytic cleavage released the active drug into the cytoplasm. In a similar study Monsigny *et al.* (40) were able to demonstrate the targeting of methotrexate by conjugation to fucosylated neoglycoproteins. These conjugates resulted in a more efficient killing of tumor cells by methotrexate.

As previously indicated, before a carrier can be effective, the link between drug and carrier must remain stable in the bloodstream and withstand the action of serum hydrolases. On the other hand, unless the drug is able to act in conjugated form at the cell surface, it must be released from the carrier after interaction of the conjugate with the target cell. In addition, its mode of release must allow the drug to reach its subcellular target and interact effectively with it. Because the most general fate of molecules bound by surface receptors is to be internalized by endocytosis, and conveyed to lysosomes for digestion, an obvious way of insuring appropriate release of drug is to rely on lysosomal hydrolysis. This approach is of course limited to drugs that are not inactivated in the lysosomes and that can reach their biochemical target from the lysosomal compartment. Monsigny *et al.* have demonstrated that the stability of daunorubicin-carrier conjugates in serum can be enhanced by incorporating peptide spacers between this drug and its carrier (41). These "drug-arm-carrier" conjugates can be specifically cleaved by lysosomal proteases leading to the release of active drug inside target cells. Moreover, these conjugates were more effective than free drug in killing of tumor cells. Similar results were obtained by Trouet *et al.* (16) using oligopeptide spacers varying in length from one to four amino acids. In their studies, the direct conjugate between daunorubicin and carrier was resistant to hydrolysis by lysosomal enzymes and drug was not released intracellularly in an active form. Hydrolysis by lysosomal enzymes and subsequent drug activity was, however, increased following introduction of a tetrapeptide spacer. The tetrapeptide conjugates remained stable in the presence of serum, as required for authentic lysosomotropic drug-carrier complexes, and the chemotherapeutic activity of daunorubicin increased with the carrier's sensitivity to lysosomal hydrolysis. Similar augmentation in the therapeutic index of primaquine was observed in both extraerythrocytic and hepatic stages of murine malaria following linkage of this antimalarial agent to a hepatocyte-targeted glycoprotein via a tetrapeptide spacer (42).

A.1.2.1 Glycoprotein targeting of antivirals

Fiume *et al.* (43) have demonstrated that galactosylated serum albumin conjugated to adenine arabinoside (ara-a) and asialofetuin-ara-a conjugates, administered to mice with ectromelia virus-induced hepatitis, were equally effective in inhibiting virus DNA synthesis in liver without producing significant inhibition of cellular DNA synthesis in intestine and bone marrow. Similar results were observed with ara-c and trifluorothymidine (35-38,44). These findings were, in part, due to selective hepatocyte uptake which in turn reduced the whole animal dosage required for a therapeutic effect (*i.e*, ten-times less conjugated ara-a was required to inhibit viral DNA synthesis as compared to free ara-a). Galactosylated serum albumin has a significant advantage over asialofetuin as a hepatotropic carrier since conjugates prepared with homologous albumin are not immunogenic (43).

A.1.2.2 Glycoprotein targeting of immunostimulants

A number of synthetic immunostimulants are active *in vitro*, however, due to pharmacological problems, their *in vivo* activity is minimized. One approach to overcome this problem has been to conjugate immunostimulants to glycoproteins or neoglycoproteins. For example, Monsigny *et al.* (45) and Roche *et al.* (46) have shown that MDP-conjugated mannosylated neoglycoproteins can be targeted to alveolar macrophages. These macrophages become tumoricidal and mice and rats are protected against metastatic growth of Lewis lung carcinoma. MDP-conjugated mannosylated neoglycoprotein are several orders of magnitude more effective than free MDP in activating macrophages and protecting animals. Recently, Monsigny has conjugated MTP to polylysine partially substituted with gluconyl groups. These conjugates are even better than the neoglycoprotein conjugates in potentiating macrophage activity and are currently in phase I clinical trials in France (see Methods section below).

A.2 Combination antiviral therapy

Experience with anticancer chemotherapeutic agents has clearly demonstrated that combination therapy is often more effective than the additive effects of individual agents. This is particularly apparent when the mechanisms of action of drugs are different. As the number of antiviral agents increases, it is reasonable to believe that similar combination therapy will be effective in viral diseases. Combination therapy offers a distinct advantage over single agent therapy in that the therapeutic dose can be reduced and toxicity minimized. Moreover, the number of potential failures or recurrences due to the selection and overgrowth of drug resistant virus mutants can be limited. A number of observations on antiviral drug combinations with either additive or synergistic effects have been reported (47-55). For example, Fischer *et al.* have demonstrated the *in vitro* synergistic effects of 5'-amino-thymidine and iododeoxyuridine against herpes simplex virus (49). Ayisi *et al.* have shown that combinations of 5-methoxymethyl-deoxyuridine with either vidarabine or phosphonoacetic acid are synergistic against herpesviruses in cell culture (48). In

addition, vidarabine in combination with phosphonoacetic acid has been found to produce a synergistic response against herpesviruses and retroviruses *in vitro* (51) and against herpesviruses *in vivo* (52). Combinations of acyclovir with either vidarabine, vidarabine monophosphate or phosphonoformic acid have been reported to produce additive antiviral effects both *in vitro* and *in vivo* (53,54). Similar enhancement of activity has been achieved with combinations of antiviral agents against human influenza viruses *in vitro* (50). The combination of ribavirin with either amantadine or rimantadine hydrochloride resulted in a significantly enhanced antiviral effect against a several subtypes of influenza A viruses. Recently, combination therapy with azidothymidine and recombinant interferon alpha resulted in synergistic inhibition of human immunodeficiency virus replication (55).

B. OBJECTIVES AND APPROACH

B.1 Phase I studies

B.1.1 Broad spectrum activity of liposome-encapsulated drugs

Our previous studies have demonstrated the effectiveness of liposomes as carriers of antiviral and immunostimulating drugs in the treatment of herpes- and arenavirus infections. The general acceptance of liposomes as carriers of antiviral and immunostimulating drugs will depend, in part, on the demonstration of their effectiveness in a variety of viral diseases. Thus, we will extend our previous DOD contract studies to include additional murine models of diseases of interest to the military, *i.e.*, the bunyavirus, Punta Toro, and the flavivirus, Banzi. We will also study murine models of retrovirus-induced immunosuppression (e.g. Rauscher leukemia and LP-BM5 virus-induced murine AIDS). These viruses were selected because of their different tissue-tropisms (*i.e.*, Punta Toro for liver, Banzi for brain and Rauscher and MAIDS for lymphoid cells). In addition, Punta Toro and Banzi viruses result in acute infections whereas Rauscher and LP-BM5 viruses result in chronic persistent disease.

The effects of chemotherapeutic and immunotherapeutic agents will be assessed by measuring mean survival time, viremia, and/or virus replication in selected organs. Survivors will be examined for virus specific serum antibody and resistance to virus rechallenge. The Punta Toro and Banzi virus models are currently in use in our laboratory. Rauscher leukemia and LP-BM5 viruses have been used as models for screening of antivirals effective against AIDS, a disease of worldwide significance. Studies on these viruses have been conducted in collaboration with Dr. Erik De Clercq of the Rega Institute, Leuven, Belgium. One of us (JDG) spent part of his sabbatical (1987-1988) in Dr. De Clercq's laboratory during which time he gained experience with several retroviral models of immunosuppression. Both the murine AIDS and Rauscher leukemia models are now established in our laboratory.

Our initial studies have focused on ribavirin and MTP-PE since these drugs were effective in our other models. However, subsequent studies will include additional antivirals and/or immunostimulants. The selection of these antivirals and immunostimulants will be made in consultation with the contracting officer.

B.1.2 Selective drug targeting by neoglycoproteins

We have demonstrated in studies supported by previous DOD contracts that liposomes are useful vehicles for the delivery of antivirals and immunostimulants. However, these vehicles have some limitations as targeting agents in that they are compartmentalized primarily by the reticuloendothelial system. Therefore, a second targeting approach has employed conjugation of drugs to neoglycoproteins. This approach has allowed us to deliver drugs to more specific sites of viral infection. Initially, our studies have focused on ribavirin and MTP-PE since these have proven effective following liposome encapsulation. Subsequent studies will employ additional antivirals and immunostimulants which will be selected in consultation with the contracting officer.

One of our goals for the first year of this contract was to determine the effectiveness of conjugated drug delivery in the treatment of virus infections which occur at different tissue sites. Future studies will compare the relative effectiveness of different targeting approaches using both murine and guinea pig models of viral diseases described in our contract proposal. Successful therapeutic approaches will then be extended to the nonhuman primate models of influenza and Punta Toro previously described.

Homologous serum albumin with added terminal mannose or galactose residues will be conjugated with drugs to target them to macrophages and hepatocytes, respectively. Drug conjugates will be chemically characterized to determine the amount of drug per carrier molecule and biologically characterized to determine functional activity. Conjugation of antivirals with galactosylated albumin should provide a more efficient delivery to infected hepatocytes while conjugation of immunostimulants to mannosylated albumin should enhance the uptake by macrophages and monocytes. Dr. Monsigny from the Universite' d'Orleans, Centre de Biophysique Moleculaire du Centre National de la Recherche Scientifique, Orleans, France, will be collaborating with us in the preparation and characterization of drug-conjugated proteins. Dr. Monsigny has had extensive experience in conjugating drugs such as muramyl dipeptide and tripeptide to neoglycoproteins. In addition, he has recently developed a delivery system consisting of poly-lysine partially substituted with gluconyl groups which is more a more effective carrier than serum albumin. This carrier has recently received approval by the French medical authorities for use in man.

B.1.3 Combination antiviral/immunostimulant therapy

A number of newly developed drugs show promising antiviral activity *in vitro*. However, *in vivo*, many of them cause adverse side effects including damage to components of the immune system. Thus, while

antivirals may reduce viral burdens in target organs, a host with a compromised immune system may not effectively remove residual virus. Therefore, any damage to immune components should be compensated for during antiviral chemotherapy. Our approach to this problem will be to attempt to balance the immunosuppressive effects of antivirals with administration of immunostimulants. This strategy has the added advantage that immunostimulants may also act synergistically in those situations where antivirals have minimum or no immunosuppressive effects. Our preliminary data (Contract DAMD 17-84-C-4144 and J. Cell Biology, Supplement 12B, 1988, Abstract W-102 page 255) clearly indicates that combination antiviral/ immunostimulant therapy is indeed effective in the treatment of herpesvirus and arenavirus infections.

Most immunostimulants possess a unique set of immunomodulating features and provide varying degrees of benefit to the infected host depending on the tissue site and degree of virus infection. For example, some immunostimulants activate macrophages and induce high levels of interferon, whereas others activate macrophages but are poor inducers of interferon. Thus, the judicious selection of an immunostimulant to be used in combination therapy with an antiviral requires 1) an understanding of the biological effects of the immunostimulant on the components of the immune system, 2) a knowledge of the mode of action of the antiviral agent, and 3) an understanding of the immune response to the virus infection.

Initially our attention will focus on combination therapy using ribavirin and MTP-PE in murine, guinea pig and primate models of disease since the effects of these drugs have already been characterized in our earlier studies. As other promising antivirals and immunostimulants are developed and characterized under DOD contracts, we will attempt to enhance their efficacy either alone or in combination by targeting using liposomes, or neoglycoproteins. In these studies we will select immunostimulants which have a broad spectrum effect on both specific and nonspecific components of the immune system. Murine, guinea pig and primate models will be used to evaluate therapeutic potential of selected drugs.

B.2 Phase II studies

B.2.1 Preclinical evaluation of liposome-encapsulated ribavirin and MTP-PE

In our previous studies we have shown that encapsulation of ribavirin and/or MTP-PE into liposomes resulted in improved targeting and therapeutic effectiveness when examined in both murine and guinea pig models of viral disease. While these results are encouraging, they do not necessarily ensure the usefulness of liposome-encapsulated drugs in viral diseases of man. A more reliable predictor of clinical usefulness should come from studies performed in nonhuman primates. Thus, in this renewal we propose to study whether targeting of antivirals and/or immunostimulants will enhance the therapeutic efficacy of these drugs in viral diseases of animal species more closely related to man (squirrel and African green monkeys). Since the

liver and lung appear to be important target organs in the pathogenesis of several virus infections, we will focus our attention on infections occurring in these organs.

One of the viruses to be examined in these animals will be influenza. In this model animals will be challenged intranasally with virus and examined for fever and virus shedding. Antivirals and immunostimulants will be administered individually or in combination as well as in free or liposome-encapsulated form. Since free ribavirin has been shown to be effective in the therapy of influenza in squirrel monkeys, our studies with this drug will be designed to improve the therapeutic index of this drug via targeted delivery and/or combination with an immunostimulant.

The hepatotropic Punta Toro virus will serve as a second model of viral disease. In this model, African Green monkeys will be challenged with virus and treated with free or liposome-encapsulated antivirals and/or immunostimulants. Animals will be examined for viremia and hematological and liver enzyme changes.

B.2.2 Timing of Phase II studies

Phase I studies will allow us to compare the efficacy of liposomes and neoglycoproteins as targeting vehicles for antivirals and/or immunostimulants in various viral disease models. These models provide us with an opportunity to examine the effectiveness of targeted drug delivery in both acute and chronic virus infections in which the primary sites of replication or dissemination vary. Phase II studies will allow us to test targeting approaches, proven to be effective in Phase I studies, in nonhuman primate models of viral disease. These studies should enable us to more accurately predict their clinical potential in man.

Our initial studies using liposomes as a targeting vehicle for ribavirin and/or MTP-PE have already proven successful in several of the murine and guinea pig models of disease. Thus, we will begin Phase II studies using liposome-encapsulated ribavirin and/or MTP-PE immediately. When other targeting vehicles and/or drugs prove successful in Phase I studies they will then be tested in Phase II studies. In this way Phase I and Phase II studies will be conducted concurrently throughout the contract period.

C. RESULTS

The second year of this contract has been devoted to studies aimed at further characterizing the immunological profiles of three drugs, Poly I:C-LC, CL 246738 and Riker-3M compound. These studies were designed to examine the possible detrimental effects of multiple drug dosage, using drugs which have proven to be effective in our primary screen when given in a single dose. These studies were prompted by the concern that multiple dosages of a drug may result in acute toxicity. Therefore, it was decided that experiments aimed at evaluating the effects of varying dosages of an immunomodulator on resistance to viral infection and changes in immunological functions should be planned. In particular, the effects of multiple drug dosages on resistance to Banzi virus challenge, interferon induction, RES stimulation, and NK cytotoxicity were examined. We have also evaluated the carrier potential of liposomes in the delivery of immunomodulating agents which will soon enter clinical trials at USAMRIID. Since Poly I:C-LC is a prime candidate for initial clinical trials, we performed a series of experiments to characterize the therapeutic advantage of liposome delivery of this drug.

Studies performed in the second year of this contract have also included 1) the development of a Dengue 2 virus model, 2) the evaluation of interferon responses in primates treated with free and liposome encapsulated Poly I:C, 3) a comparison of the efficacy of Poly I:C-LC and dextran-Poly I:C conjugates, and 4) an evaluation of liposome encapsulated AVS-5587. In addition, our subcontractor, Dr. Monsigny, has prepared AVS-5587 conjugated to mannosylated poly-L-lysine for evaluation in macrophage functional studies and in *in vivo* resistance.

C.1 Evaluation of Poly I:C-LC, Riker-3M compound and CL 246738

C.1.1 Serum Interferon

Table 1 illustrates the serum interferon levels in mice receiving oral doses (4 mg/ms) of Cl 246738 every 3 days for 12 days. Note that one day following the first gavage, mice had significant levels of circulating interferon in their serum. When examined again one day after each of their subsequent gavages, the interferon levels were only slightly elevated suggesting that multiple drug treatment may result in a hyporesponsive state. It is appreciated that this result was obtained using a single dose (recommended by the Project Officer) which induces the maximum degree of macrophage cytotoxicity (56) although lower doses are optimal for NK cell activation and interferon induction (57). It is possible that the suppressive acitivity of the drug in multiple doses was due to the super-optimal dose used in these experiments. Similar results were observed with the Riker 3-M compound, R837 (Table 2). It should be pointed out that these results were obtained using a single dose of the drug and a single point of serum sampling. It has been reported that in guinea pigs receiving 3 mg/kg of this drug, maximum level of serum interferon is observed at 12 hours after treatment although significant levels persist at 24 and 72

hours (58). It is likely that 24 hours post treatment is not the optimal time in mice and may explain the very low levels of interferon (comparable to control levels) in mice receiving multiples doses of the drug.

C.1.2 Natural Killer Cell Cytotoxicity

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M compound (10 mg/kg) 2 days prior to evaluation of NK cytotoxicity. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. As illustrated in Table 3, all groups showed significantly elevated NK cytotoxicity at all effector to target cell ratios. However, multiple doses appeared to result in a slight diminution of NK cell activity. Thus, while multiple doses of Riker-3M compound did not grossly effect NK cell activity they may have had some slight adverse effects.

A similar protocol was used with CL 246738. Similar to our observations with Riker-3M compound, multiple doses appeared to enhance NK cytotoxicity; however, a slight diminution of this activity occurred following multiple doses (Table 4).

C.1.3 Macrophage cytotoxicity

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M compound (10 mg/kg) 2 days prior to evaluation of macrophage cytotoxicity. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. An additional group of mice was injected with *Propionibacterium acnes* (70 mg/kg) as a positive control. As illustrated in Table 5 no significant macrophage cytotoxicity was observed in any of the groups. Macrophages from mice injected with *P. acnes* exhibited the expectected anti-tumor cytotoxic activity (data not shown). Similar results were obtained with CL 246738 (Table 6).

C.1.4 Effects on the reticuloendothelial system

The data in Table 7, shows that while one dose of CL 246738 was stimulatory, two and three doses of the drug had a somewhat reduced effect of RES activity. Surprisingly, four doses also was stimulatory. Currently we have no explanation for this anomaly. Similar results were obtained with Riker-3M compound (Table 8).

C.1.5 Phagocytosis

Mice were divided into 4 groups. Group 1 received a single gavage with Riker-3M compound (10 mg/kg) 2 days prior to evaluation of phagocytic activity of peritoneal cells. Group 2 received gavage on days 2 and 4; Group 3 on days 2, 4, and 6; and Group 4 on days 2, 4, 6 and 8. As illustrated in Figure 1 when drug was administered two days prior to assay, a significant enhancement in phagocytic activity was observed. However, multiple drug doses did not result in sustained phagocytic activity (Figures 2 - 4).

C.1.6 Resistance to Banzi virus following multiple injections with Riker-3M compound or CL 246738

Mice were divided into 5 groups. Group 1 received a single gavage with Riker-3M compound (10 mg/kg) two days prior to virus challenge; Group 2 received gavage 4 days prior to challenge; Group 3 received gavage 6 days prior to challenge; Group 4 received gavage 8 days prior to challenge; and Group 5 received gavage 10 days prior to challenge. Figure 5 illustrates that animals in Group 1 were able to resist virus lethality (60% protection) while animals in Group 2 were only marginally protected (30% protection). None of the other three groups were protected.

A similar protocol was used for CL 246738. Except for animals which received drug 10 days prior to virus challenge (Group 5) significant protection was observed in each of the other groups (Figure 6). Note the time dependency of drug administration and level of protection. Moreover, animals treated with CL 246738 were more resistant than those treated with Riker-3M compound.

C.1.7 Resistance to Banzi virus following multiple injections with Poly I:C-LC

Mice were divided into 11 groups in which the following treatment protocols were examined. These treatment protocols were selected to complement ongoing studies at Ft. Detrick under the direction of Dr. Meir Kende.

Group	Day of Poly I:C-LC Injection (i.v. 1 mg/kg)
A.	-20, -15, -10, -5, 0, +5, +10 (saline i.v. control)
B.	-20, -15, -10, -5, 0, +5, +10
C.	-10, -5, 0, +5, +10
D.	-11, -6, -1,+4, +9
E.	-12, -7, -2,+3, +8
F.	-13, -8, -3, +2, +7
G.	-14, -9, -4, +1, +6
H.	-21, -16, -11, -6, -1, +4, +9
I.	-22, -17, -12, -7, -2, +3, +8
J.	-23, -18, -13, -8, -3, +2, +7
K.	-24, -19, -14, -9, -4, +1, +6

As illustrated in Figures 7a and 7b, groups B, D, E, G, H, and K were able to resist challenge with Banzi virus. In contrast groups F (Figure 7a), J, and I (Figure 7b) were not protected and mice died at about the same time as saline controls. We have no explanation as to why mice in groups F, J, and I were still fully susceptible to Banzi infection especially since their therapy differed by only one day (compare group F with groups E and G in Figure 7a, and groups I and J with groups H and K in Figure 7b). In general, however, it appears

that multiple dosages have no deleterious effects on resistance to Banzi virus infection.

C.2 Evaluation of Dengue 1 virus (West Pac) infection of squirrel monkeys

Dengue 1 virus infection in squirrel monkeys, which were confirmed to be seronegative prior to infection was evaluated. Monkeys were bled prior to virus infection and serum samples were evaluated for the presence of neutralizing antibody. None of the animals had detectible antibody levels. These animals were inoculated intramuscularly (0.5 ml) into their forearms with an undiluted lyophilized preparation of Dengue 1 (West Pac 74) containing 1 X 10^5 plaque forming units per ml. Fever was monitored and serum samples collected daily from each monkey for 12 days. In addition, differential counts and liver enzyme changes were evaluated. As illustrated in Table 9, no change in temperature or liver enzymes were observed at any time following infection and only a slight viremia was evident. This viremia was slightly higher at day 5 than at day 4.

C.3 Targeting of Poly I:C and 7-thia-8-oxoquanosine (AVS-5587)

C.3.1 Liposome encapsulation of Poly I:C and AVS-5587

These experiments were designed to evaluate the effect of administering the immunomodulator poly I:C or the antiviral AVS-5587 in a carrier which should release the drugs slowly and hence sustain their concentration in the body (circulation) for a longer period. Also, encapsulation should protect the drug from the detoxifying effects of host enzymes. These drugs were administered intravenously to achieve the maximum blood level quickly. As illustrated in Table 10, the encapsulation efficiency of poly I:C was quite good. Thus, one hour after preparation, 50% of the drug could be shown to be associated with liposomes. Moreover, this association was stable over a 36 hour period as illustrated by the fact that 40% was still liposome-associated. Encapsulation of AVS-5587 was not as good as that observed for poly I:C; however, 30% of the drug was liposome associated at one hour and 20% at 36 hours following the initial preparation. The data suggest that both poly I:C and AVS-5587 can be easily incorporated into freeze dried liposomes made from synthetic lipids (CIBA-GEIGY). Liposomes loaded with these drugs are stable over a 36 hour period.

C.3.2 Interferon induction by free and liposome-encapsulated Poly I:C

Table 11 illustrates the serum interferon titers in mice inoculated intravenously with either free or liposome-encapsulated Poly I:C at eight hours post-injection. Note that liposome-encapsulated poly I:C (589 IU) was as good as free poly I:C (437 IU) in inducing interferon. Moreover, the kinetics of interferon response revealed that liposome-encapsulated poly I:C was as good or even slightly better than

free Poly I:C when examined at 12 and 24 hours post inoculation (Table 12).

C.3.3 Natural killer cell activity following administration of free or liposome-encapsulated Poly I:C

Natural killer cell activity was examined at 1, 2 and 3 days following the intravenous administration of drug (Table 13). Note that both free and liposome-encapsulated poly I:C (50 μg/kg) were good enhancers of NK activity on any of the three days examined and no significant difference between the two drug formulations was observed. In addition, both appeared to have maximal effects at two days post infection.

C.3.4 Effects of liposome encapsulation of Poly I:C on reticuloendothelial system function

Table 14 represents a composite of 3 clearance experiments. As illustrated, liposome-encapsulation significantly enhanced Poly I:C's ability to stimulate the reticuloendothelial system as measured by the increased rate of labelled SRBC clearance (T/2). This effect was demonstrated at dosages of 500, 50 and 5 μg/kg.

C.3.5 Effects of liposome encapsulation of Poly I:C on macrophage cytotoxicity

Table 15 illustrates the cytotoxic potential of macrophages stimulated with free or liposome-encapsulated poly I:C. Note that our results are variable; nonetheless, a positive effect for liposome-encapsulated drug was observed on at least one occasion (exp. #3; cytotoxicity = 18 for free and 82% for encapsulated drug). A second experiment (#2) also indicated a positive effect, but the sham liposome effect invalidated this experiment. Based on these observations, it is impossible to draw any conclusion on the effectiveness of liposome encapsulated poly I:C in activating cytotoxic macrophages.

C.3.6 Effects of free or liposome-encapsulated Poly I:C on splenic lymphocyte subpopulations

No significant differences in splenic cell populations were noted following the administration of either liposome-encapsulated or free poly I:C (Table 16).

C.3.7 Effects free or liposome-encapsulated Poly I:C on phagocytic activity of peritoneal macrophages

A number of experiments were performed to analyze the phagocytic activity of PEC from poly I:C treated mice. As illustrated in Figures 8 and 9, a slight increase in phagocytic activity was sometimes observed following treatment with liposome versus free poly I:C. However, this effect was not always seen (Figures 10 and 11). Thus,

liposome-encapsulated Poly I:C at the dose examined had little, if any, effect on phagocytosis by peritoneal macrophages.

C.3.8 Protection against Banzi virus infection following administration of liposome-encapsulated Poly I:C

Figures 12-15 illustrate the protection afforded mice following poly I:C administration. Note that liposome-encapsulated drug was as effective and in some instances more effective than free drug in protecting mice from lethal Banzi virus challenge. Quite surprisingly, as little as 5 or 0.5 μg/kg was effective.

C.3.9 Interferon induction by free or liposome-encapsulated Poly I:C in primates

In an effort to determine whether selective delivery of Poly I:C to macrophages would enhance the interferon response, we inoculated squirrel monkeys intravenously with 0.2 mg/kg free or liposome encapsulated Poly I:C. One day following inoculation animals were bled and serum interferon levels were determined by a neutral red dye exclusion assay.

Table 17 illustrates the serum interferon levels 24 hours after drug administration. As indicated, no interferon was observed in pre-bleed samples and none was induced following administration of empty liposomes (Group 1, sham). In contrast, liposome encapsulated Poly I:C was a potent interferon inducer (Group 4). The encapsulated drug (Group 4) was even better than free Poly I:C (Group 2) or the Poly I:C-LC derivative (Group 3). Similar results were obtained in both experiments 1 and 2.

Monkeys were also inoculated with Poly I:C-dextran which was obtained from Dr. Bellows. At 24 hours following intravenous administration, a significant serum interferon response was observed (Table 17, Group 5). This response was greater than that observed with free Poly I:C or Poly IC-LC but was not as great as that observed with liposome encapsulated Poly I:C (compare Group 5 with Groups 2,3, and 4 in Table 17).

C.3.10 Interferon response to free or liposome encapsulated AVS-55587

Groups of three mice were inoculated, intravenously, with 10 mg/kg AVS-5587 either in a free or liposome encapsulated form. This was the highest dose of drug which could be administered due to solubility problems. Mice were bled at 4, 12, and 24 hours post inoculation and examined for serum interferon. Only low levels of interferon were observed at 4 and 12 hours post administration and no differences were noted between free and encapsulated drug (Table 18).

C.3.11 Stimulation of the reticuloendothelial system by free or liposome encapsulated AVS-5587

As illustrated in Table 19, neither free nor liposome encapsulated AVS-5587 (10 mg/kg) stimulated the reticuloendothelial system as measured by the clearance rate of ($T_{\frac{1}{2}}$) injected labeled SRBC from the circulation.

C.3.12 Macrophage cytotoxicity following administration of free or liposome-encapsulated AVS-5587

Table 20 illustrates the lack of macrophage cytotoxicity following intraperitoneal inoculation of 50 mg/kg of AVS-5587. A higher concentration of drug was used this experiment due to the fact that we were able to inoculate mice with a larger volume intraperitoneally. Note that neither free nor liposome encapsulated drug was able to render peritoneal macrophages cytotoxic. In contrast *P. acnes* was highly effective.

C.3.13 Peritoneal exudate cell phagocytosis following treatment with free or liposome-encapsulated AVS-5587

Figures 16 and 17 illustrate the phagocytic activity of peritoneal exudate cells from control mice and mice treated with AVS-5587. As illustrated in both figures neither oral nor intraperitoneal administration of drug had any effect on phagocytic activity of peritoneal cells. The oral route was employed because of published reports indicating that this route was effect in resistance to virus infection.

We also tested whether liposome encapsulation might increase the ability of AVS-5587 to augment peritoneal cell phagocytosis. Note that liposome encapsulated drug given 2 days before testing had no effect on drug activity (Figure 18). The lack of an effect was not due to the dose employed since increasing drug concentration five fold had no effect. Similar results were obtained with free or encapsulated drug (Figures 19 and 20).

C.3.14 Effects of free or liposome-encapsulated AVS-5587 on lymphocyte subpopulations

Table 21 indicates that AVS-5587 had no effect on the proportion of B and T cells in the spleen or the absolute numbers of these cells. We are uncertain as to the biological significance of the apparent splenomegaly which occurred following administration of the $NaHCO_3$ vehicle.

C.3.15 Effects fee or liposome-encapsulated AVS-5587 on NK cytotoxicity

Table 22 illustrates the inability of AVS-5587 to augment splenic NK cytotoxicity. Note that neither free nor liposome encapsulated drug was effective at any of the three Effector:Target cell ratios employed.

C.3.16 Resistance to Banzi virus following administration of free or liposome-encapsulated AVS-5587

While the *ex vivo* interferon and immunological function studies described above suggest a lack of immunostimulatory activity by AVS-5587, some enhancement of resistance to Banzi virus infection was observed (Figures 21 and 22). Note that while both free and liposome encapsulated drug were equally effective in preventing death when administered one day before infection (Figure 21), liposome-encapsulated drug was slightly better when administered on the day of infection (Figure 22). In addition, both free and encapsulated drug were equally effective in prolonging mean survival times when given one day before infection (Figure 21).

C.4 Dengue 2 infection in squirrel monkeys

Since our initial attempts to develop a dengue squirrel monkey model using the Type 1 dengue virus (West Pac) provided by Dr. Ken Eccles (WRAIR) were unsuccessful, we initiated studies using a Type 2 dengue virus (strain 16681) provided by Dr. T. Monath. Monkeys were screened for anti-dengue antibodies prior to being included in this study. Animals were examined daily for five days prior to virus inoculation for baseline values for fever and white blood cell counts. Monkeys were inoculated subcutaneously with Dengue 2 (1×10^6 p.f.u.; strain 16681), provided by Dr. Tom Monath. The animals were bled each day following virus inoculation and monitored for liver enzyme changes, fever and morbidity. In addition, the presence of infections virus in blood was measured by plaque titration on mosquito cells.

As shown in Table 23 all three virus infected monkeys were found to have significant viremia one to two days following infection. Note however, that from days 3 through 7 all three animals had persistent viremia although no obvious peak was observed. It is unlikely that this represents residual virus since non cell associated viruses generally do not persist in the circulation for prolonged periods. Furthermore, all of the infected monkeys developed anti-dengue antibodies (titer >160) by 60 days post infection, as reported by the serology division at USAMRIID.

No statistically significant differences in temperature was observed at any time following virus inoculation in any of the infected monkeys (Table 23). Slides were also prepared for differential leukocyte counts; these data will be reported at a future date.

Table 24 summarizes the liver enzyme data. The most obvious difference noted was that one of the infected monkeys (# 921) had a 4 - 5 fold increase in Gamma GTP on days 1-6 following infection. This same monkey also had elevated total bilirubin on days 1 - 6 and presented with the highest viral titers in blood (see Table 23). While there was a trend toward increasing SGOT and SGPT levels on days 2 - 6 in infected monkey #76, the significance of this finding is unclear. No obvious differences were noted in the LDH or alkaline phosphatase levels.

D. CONCLUSIONS

1. Multiple doses of Riker-3M compound or CL 246738 did not result in sustained augmentation of serum interferon levels and natural killer cell activity. While augmentation was readily apparent after a single dose, multiple doses of Riker-3M compound or CL 246738 may have a adverse effects on these functions.

2. Neither single nor multiple doses of Riker-3M compound or CL 246738 were effective in augmenting macrophage cytotoxicity.

3. While a single dose of Riker-3M compound significantly enhanced phagocytosis, this effect was not sustained following multiple drug doses.

4. Both Riker-3M compound and CL 246738 were effective in enhancing resistance to banzivirus encephalitis when administered several days prior to virus infection. However, animals treated with CL 246738 were more resistant than those treated with Riker-3M compound.

5. Multiple doses of Poly I:C-LC had little , if any, detrimental effects on resistance to banzi virus encephalitis.

6. Multiple doses of CL 246738 or Riker-3M compound had no significant detrimental effects on the clearance rate of radiolabeled erythrocytes from the circulation.

7. Insignificant viremia nad liver enzyme changes were observed in squirrel monkeys infected with the West Pac strain of Dengue 1.

8. Liposome encapsulation of poly I:C enhances the therapeutic potential of this drug. This enhancement may result from selective macrophage delivery and subsequent stimulation of the reticuloendothelial system.

9. Poly I:C-Dextran appears to be as good an interferon inducer in squirrel monkeys as is Poly I:C-LC.

10. AVS-5587 was not as effective as other drugs (*i*.e. Poly I:C-LC, Riker-3M compound or CL 246738) in our functional assay. In

addition neither liposome encapsulation nor conjugation to mannosylated polymers was able to boost the activity of this drug.

11. The Dengue 2 virus used in these studies appeared to be infectious in squirrel monkeys. However, the presence of a an arbovirus contaminant in this seed stock has been suspected. Future studies will employ plaque purified virus free of contaminants.

E. FUTURE DIRECTIONS

Based on the data obtained during the second year of this contract period we feel that future studies should focus on the immunomodulatory activity of Poly I:C-LC, Riker-3M compound and CL 246738 in non human primates (squirrel monkeys). These studies should include encapsulation of drugs in liposomes as a means to potentiate their activity and reduce their toxicity. The continued development of a Dengue 2 virus model in squirrel monkeys will provide the opportunity to evaluate drug induced resistance to an arboviral infection. These studies will provide the preclinical data required for approval for the use of immunomodulating drugs in man.

LITERATURE CITED

1. Hillyard, I.W. In Ribavirin: A Broad Spectrum Antiviral Agent (R.A. Smith and W. Kirkpatrick, eds.), p 59, Academic Press, New York, 1980.

2. Canonico, P.G. In Antibiotics (F.E. Hahn, ed), p161, Springer Verlag Publ. Co., 1980.

3. Trouet, A., Deprez-De Campeneere, D. and de Duve, C. Nature (London) New Biol. 239:110, 1972.

4. Deprez-De Campeneere, D. and Trouet, A. Eur. J. Cancer 16:981, 1980.

5. Rustum, U.M., Dave, C., Mayhew, E. and Papahadjopoulos, D. Cancer Res. 39:1390, 1979.

6. Gregoriadis, G. and Neerunjun, E.D. Biochem. Biophys. Res. Commun. 65:537, 1975.

7. Ghose, T. and Blair, A.H. J. Natl. Cancer Inst. 61:657, 1978.

8. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R. and Sela, M. Cancer Res. 35:1175, 1975.

9. Vitetta, E.S. and Uhr, J.W. Ann. Rev. Immunol. 3:197, 1985.

10. Kaneko, Y. Horm. Met. Res. 13:110, 1981.

11. Varga, J.M., Asato, N., Lande, S. and Lerner, A.B. Nature (London) 267:56, 1977.

12. Lynch, W.E., Sartiano, G.P. and Ghaffar, A. Amer. J. Hematol. 9:249, 1980.

13. Monsigny, M., Kieda, C., Roche, A.C. and Delmotte, F. FEBS Lett. 119:181, 1980.

14. Kitao, T. and Hattori, K. Nature (London) 265:81, 1977.

15. Ryser, H.J-P. and Shen, W-C. Proc. Natl. Acad. Sci. USA 75:3867, 1978.

16. Trouet, A., Masquelier, M., Baurain, R. and Deprez-De Campeneere, D. Proc. Natl. Acad. Sci. USA 79:626, 1982.

17. Poste, G. and Papahadjopoulos, D. Proc. Natl. Acad. Sci. USA 73:1603, 1976.

18. Alving, C.R. In Targeting of Drugs (G. Gregoriadis, J. Senior and A. Trouet, eds) p 337, Plenum, New York, 1982.

19. Koff, W.C., Showalter, S.D., Hampar, B. and Fidler, I.J. Science 228:495, 1984.

20. Gangemi, J.D., Nachtigal, M., Barnhart, D., Krech, L. and Jani, P. J. Infect. Dis. 155:510, 1987.

21. Bangham, A.D., Standish, M.M. and Watkins, J.C. J. Mol. Biol. 13:238, 1965.

22. Papahadjopoulos, D., Miller, N. Biochem. Biophys. Acta 135:624, 1967.

23. Airian, G., Huang, L. Biophys. J. 25:A292, 1979.

24. Szoka, Jr., F. and Papahadjopoulos, D. Proc. Natl. Acad. Sci. 75:4194, 1978.

25. Fidler, I.J., Raz, A., Fogler, W.E., Kirsh, R., Bugelski, P. and Poste, G. Cancer Res. 40:4460, 1980.

26. Fidler, I.J., Barnes, Z., Fogler, W.E., Kirsh, R., Bugelski, P. and Poste, G. Cancer Res. 42:496, 1982.

27. Rahman, Y.E., Cerny, E.A., Patel, D.R., Lau, E.H. and Wright, B.J. Life Sciences 31:2061, 1982.

28. Ruebush, M.J., Halc, A.H. and Harris, D.T. Infect. Immun. 32:513, 1981.

29. Kramp, W.J., Six, H.B., Drake, S. and Kasel, J.A. Infec. Immun. 25:771, 1979.

30. Neurath, A.R., Kent, S.B.H. and Strick, N. J. Gen. Virol. 65:1009, 1984.

31. Smolin, G., Okumoto, M., Feiler, S., and Condon, D. Amer. J. Ophthal. 91:220, 1981.

32. Kende, M. Alving, C.R., Rill, W., Swartz, G.M. and Canonico, P. Antimicrob. Agents and Chemo. 27:903, 1985.

33. Fidler, I.J., Sone, S., Fogler, W.E. and Barnes, Z.L. Proc. Natl. Acad. Sci. USA 78:1680, 1981.

34. Sone, D. and Fidler, I.J. Cellular Immunol. 57:42, 1981.

35. Fiume, L., Busi, C. and Mattioli, A. FEBS Lett. 153:6, 1983.

36. Fiume, L., Mattiolo, A., Balboni, P.G., Tognon, M., Barbanti-Brodano, G., De-Vries, J. and Wieland, T. FEBS Lett. 103:47, 1979.

37. Fiume, L., Busi, C., Mattioli, A., Balboni, P.G., Barbanti-Brodano, G. and Wieland, T. In Targeting of Drugs (G. Gregoriadis, Sr., J. and A. Trouet, eds.), p1, Plenum Publ. Co., New York, 1982

38. Fiume, L., Busi, C., Mattioli, A., Balboni, P.G. and Barbanti-Brodano, G. FEBS Lett. 129:261, 1981.

39. Fiume, L., Busi, C. and Mattioli, A. FEBS Lett. 146:42, 1982.

40. Monsigny, M., Roche, A-C. and Midoux, P. Biol. Cell 51:187, 1984.

41. Monsigny, M., Keida, C., Roche, A-C. and Delmotte, F. FEBS Lett. 119:181, 1980.

42. Trouet, A., Baurain, R., Deprez-De Campeneere, D., Masquelier, M. and Prison, P. In Targeting of Drugs (G. Grerориadis, Sr., J. and A. Trouet, eds), p19, Plenum Publ. Co., New York, 1982.

43. Fiume, L., Mattioli, A., Busi, C., Spinosa, G. and Wieland, T. Experientia 38:1087, 1982.

44. Balboni, P.G., Minia, A., Grossi, M.P., Barbanti-Brodano, G., Mattioli, A. and Fiume, L. Nature 264:181, 1976.

45. Monsigny, M., Roche, A-C. and Bailly, P. Biochem. Biophys. Res. Commun. 121:579, 1984.

46. Roche, A-C., Bailly, P. and Monsigny, M. Invasion Metastasis 5:218, 1985.

47. Galasso, G.J., Merigan, T.C. and Buchanan, R.A. In Antiviral Agents and Viral Diseases of Man, (G.J. Galasso, T.C. Merrigan and R.A. Buchanan, eds), p543, Raven Press, New York, 1984.

48. Ayisi, N.K., Gupta, V.S., Meldrum, J.B., Taneja, A.K. and Babuik, L.A. Antimicrob. Agents Chemother. 17:558, 1980.

49. Fischer, P.H., Lee, J.J., Chen, M.S., Lin, T-S. and Prusoff, W.H. Biochem. Pharmacol. 28:3483, 1979.

50. Hayden, F.G., Douglas, R.G. and Simons, R. Antimicrob. Agents Chemother. 18:536, 1980.

51. Shannon, W.M. and Schabel, F.M. Jr. Pharmacol. The. 11:263, 1980.

52. Gauri, K.K. Adv. Opthalmol. 38:151, 1979.

53. Oberg, B. Pharmacol. The. 19:387, 1983.

54. Schinazi, R.F., Peters, J., Williams, C.C., Chance, D. and Nahmias, A.J. Antimicrob. Agents Chemother. 22:499, 1982.

55. Hartshorn, K.L., Vogt, M.W., Chou, T-C., Blumberg, R.S., Byington, R., Scooley, R.T. and Hirsch, M.S. Antimicrob. Agents Chemother. 31:168, 1987.

56. Wong, B.S., Lumanglas, A.L., Ruszala-Mallon, V.M. and Durr, F.E. J. Immunol. 135:679, 1985.

57. Sarzotti, M., Coppenhaver, D.H., Singh, I.P., Poast, J. and Baron, S. J. Interferon Res. 9:265, 1989.

58. Chen, M., Griffith, B.P., Lucia, H.L. and Hsiung, G.D. Antimicrob. Agents Chemother. 32:678, 1988.

Table 1. Serum interferon responses in mice receiving multiple doses of CL 246738.

Day	Drug Given on Day(s)	Interferon Titer (International Units)
1	0	11,482
4	0,3	<10
7	0,3,6	117
10	0,3,6,9	14
13	0,3,6,9,12	<10

Mice were given CL 246738 (200 mg/kg), orally, on the days indicated, and bled 24 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described previously.

Table 2. Serum interferon responses in mice receiving multiple doses of Riker-3M compound.

Day	Drug Given on Day(s)	Interferon Titer (International Units)
1	0	100
4	0,3	<10
7	0,3,6	<10
10	0,3,6,9	25
13	0,3,6,9,12	<10

Mice were given Riker-3M compound (10 mg/kg), orally, on the days indicated, and bled 24 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described previously.

Table 3. Splenic NK cytotoxicity following oral administration of Riker-3M compound as a single dose or in multiple doses.

Treatment	Per cent cytotoxicity at Effector : Target Ratios		
No (days)	100:1	50:1	25:1
Control	7	2	1
1 (-2)	46*	29*	22*
1 (-4)	40*	25*	14*
2 (-2, -4)	41*	28*	16*
3 (-2, -4, -6)	31*	20*	13*
4 (-2, -4, -6, -8)	33*	20*	12*

Mice were given 200 μg of Riker-3M compound (10 mg/kg) orally either as single dose or multiple doses of 200 μg each two days apart. Two days after the last treatment, splenocytes were assayed for NK cytotoxicity. YAC cells were used as targets and 4 mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

* $p<0.05$

Table 4. Splenic NK cytotoxicity following oral administration of CL 246738 as a single dose or in multiple doses.

Treatment	Per cent cytotoxicity at Effector : Target Ratios		
No (days)	100:1	50:1	25:1
Control	28	19	12
1 (-2)	85*	81*	72*
2 (-2, -4)	77*	68*	51*
3 (-2, -4, -6)	61*	51*	35*
4 (-2, -4, -6, -8)	56*	44*	33*

Mice were given 4 mg CL 246738 (200 mg/kg) orally either as single dose or multiple doses of 4 mg each two days apart. Two days after the last treatment, splenocytes were assayed for NK cytotoxicity. YAC cells were used as targets and 4 mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

*$p<0.05$

Table 5. Lack of activation of cytotoxic macrophages by Riker-3M compound.

Treatment No (Days)	Effector to target ratio					
	40:1		20:1		10:1	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Control	62735		83237		95888	
Riker-3M Compound:						
1 (-4)	66305	(-6)	71497	(14)	83884	(12)
1 (-6)	67619	(-8)	79685	(4)	92294	(4)
1 (-8)	63299	(-1)	73515	(12)	92992	(3)
2 (-4, -6)	67555	(-8)	85534	(-3)	92506	(3)
3 (-4, -6, -8)	66340	(-6)	77725	(7)	86691	(4)

Mice were given 200 ug Riker-3M compound (10 mg/kg) orally either as a single dose or as multiple doses of 200 ug each, two days apart. Macrophage cytotoxicity assay was performed four days after the last treatment. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4x10^3$ P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^3H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 6. Lack of activation of cytotoxic macrophages by CL 246738.

Treatment No (Days)	Effector to target ratio					
	40:1		20:1		10:1	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Control	60055		67064		78434	
CL 246738:						
1 (-4)	55972	(7)	71702	(-7)	81878	(-4)
1 (-6)	44661	(26)	58155	(13)	73849	(6)
1 (-8)	48813	(19)	59116	(12)	76994	(2)
2 (-4, -6)	62950	(-5)	64765	(3)	65913	(16)
3 (-4, -6, -8)	58709	(2)	68166	(-2)	74789	(5)

Mice were given 4 mg CL 246738 (200 mg/kg) orally either as a single dose or as multiple doses 4 mg each, two days apart. Macrophage cytotoxicity assay was performed four days after the last treatment. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4x10^3$ P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with ^3H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures} - \text{CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 7. Clearance and tissue localization of SRBC following treatment with a single or multiple doses of CL 246738

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	0.0546	5.56	5.85	177	65	59
	Std. Dev.	0.0172	0.71	1.69	53	10	29
CL 246738 Day -2	Mean	0.0962	6.69	3.20	142	68	21
	Std. Dev.	0.0180	0.44	0.60	31	5	11
	P-Value	<0.01	<0.02	<0.01	NS	NS	NS
CL 246738 Day -2,4	Mean	0.0859	6.02	3.82	106	66	32
	Std. Dev.	0.0343	0.79	1.26	16	21	20
	P-Value	NS	NS	NS	<0.01	NS	NS
CL 246738 Day -2,4,6	Mean	0.0755	6.48	4.14	115	83	41
	Std. Dev.	0.0182	0.67	1.00	20	11	24
	P-Value	NS	NS	NS	<0.05	<0.05	NS
CL 246738 Day -2,4, 6,8	Mean	0.0911	6.74	3.42	93	72	26
	Std. Dev.	0.0216	0.48	0.76	17	8	12
	P-Value	<0.02	<0.02	<0.02	<0.01	NS	<0.05

Mice were given, orally, a single or multiple doses (200 mg/kg) of CL-246 738 two days apart and tested two days after the last dose for clearance of sheep erythrocytes (SRBC) from circulation and their localization in the various organs. Control mice received the same volume of pyrogen free saline. The K value represents the rate of clearance and $t_{1/2}$ represents the time to clear half of the material from the circulation. All groups were compared with the saline-treated group by the Student's T-test.

Table 8. Clearance and tissue localization of SRBC following treatment with a single or multiple doses of Riker-3M compound.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	0.0751	7.00	4.07	182	61	21
	Std. Dev.	0.0120	0.54	0.58	61	7	7
Lactic Acid Day -2	Mean	0.0927	7.73	3.26	121	72	15
	Std. Dev.	0.0073	0.37	0.26	65	8	4
	P-Value	<0.025	<0.05	<0.025	NS	NS	NS
Riker-3M Day -2	Mean	0.1044	7.67	2.91	82	74	9
	Std. Dev.	0.0118	0.28	0.33	64	3	5
	P-Value	<0.005	<0.05	<0.005	<0.05	<0.005	<0.02
Riker-3M Day -2,4	Mean	0.0889	7.43	3.39	117	68	17
	Std. Dev.	0.0053	0.76	0.21	37	13	13
	P-Value	<0.05	NS	<0.05	NS	NS	NS
Riker-3M Day -2,4,6	Mean	0.0576	6.59	5.26	196	63	43
	Std. Dev.	0.0058	0.23	0.53	31	10	20
	P-Value	<0.02	NS	<0.01	NS	NS	<0.05
Riker-3M Day -2,4 6,8	Mean	0.0834	7.23	3.67	89	75	24
	Std. Dev.	0.0141	0.67	0.65	47	13	10
	P-Value	NS	NS	NS	<0.05	NS	NS

Mice were given Riker-3M compound (10 mg/kg), orally, on the days indicated, and tested two days after the last dose for clearance of erythrocytes from circulation and their organ localization. Controls received saline or the drug vehicle, 1% Lactic acid. The K value represents the rate of clearance and $t_{1/2}$ represents the time to clear half of the material from the circulation. Groups were compared with the saline control by the Student's T-test.

Table 9. Virological and physiological changes in squirrel monkeys infected with Dengue 1 virus

Treatment	Fever	Liver Enzyme	Viremia (pfu/ml) Day Post-Infection			
			2	3	4	5
Saline Control						
#1	NC	NC	0	0	0	0
#2	NC	NC	0	0	0	0
Virus Infected						
#1	NC	NC	100	<50	125	300
#2	NC	NC	75	50	50	175
#3	NC	NC	<50	<50	75	50

Dengue seronegative squirrel monkeys were prebled and inoculated subcutaneously with 100,000 pfu of the West Pac strain of Dengue 1 virus (courtesy Dr. Ken Eccles, WRAIR). Serum samples were drawn at the days indicated and analyzed for liver enzyme changes or infectious virus in mosquito cells according to the procedure of Mr. Sherm Hasty (USAMRIID).

Table 10. Encapsulation efficiency and stability of liposomal Poly I:C and AVS-5587.

Liposome-encapsulated Drug (concentration)	Hours Post Encapsulation		
	1	24	36
Poly I:C (500 μg/ml)	50% (250 μg/ml)	45% (225 μg/ml)	40% (200 μg/ml)
AVS-5587 (5 mg/ml)	30% (1.5 mg/ml)	24% (1.2 mg/ml)	20% (1.0 mg/ml)

Liposome-encapsulated drugs were prepared by adding drugs (eg. poly I:C @ 500 μg/ml, or AVS-5587 @ 5 mg/ml) to freeze-dried synthetic lipids prepared by CIBA-GEIGY. The suspension medium consisted of Dulbecco's PBS poly I:C and 1% sodium bicarbonate for AVS-5587. Encapsulation efficiency was determined spectrophotometrically.

Table 11. Serum interferon responses in mice receiving liposome-encapsulated or free Poly I:C, eight hours post inoculation.

Inoculum	Drug Dose (μg/kg)	Interferon Titer (international units)
Sham Liposomes	--	<10
Free Poly I:C	500	437
Poly I:C/Liposomes	500	589
Poly I:C/Liposomes	50	182

Six week old mice were inoculated i.v. with free or liposomal drug and bled 8 hours later. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described in previous quarterly reports.

Table 12. Serum interferon levels in mice receiving free or liposomal Poly I:C at selected times following drug administration.

Inoculum	Interferon Titer (IU) Hrs Post-Administration		
	4	12	24
Saline	14	<10	40
Sham Liposomes	<10	<10	53
Free Poly I:C	178	74	71
Poly I:C/Liposomes	110	309	229

Six week old mice were inoculated i.v. with free or liposomal drug (500 μg/kg) and bled at the times indicated. Serum was examined for interferon using mouse L cells and the cpe neutral red assay described in previous reports.

Table 13. Splenic NK cytotoxicity following treatment with free or liposome-encapsulated Poly I:C.

Treatment	Per cent cytotoxicity		
	Day 1	Day 2	Day 3
Saline Control	16	-	-
Free Poly I:C	50	68	56
Sham Liposomes	19	14	15
Poly I:C/Liposomes	46	68	55

Mice were given Poly I:C (50 μg/kg) intravenously either in free form or encapsulated in liposomes and an NK cytotoxicity assay was performed one,two or three days thereafter. Control mice received saline or sham liposomes. YAC cells were used as targets and 3 mice per treatment group were analyzed. The assays were done at a 50:1 effector:target cell ratio and are based on the release of chromium from labeled cells. Percent toxicity was calculated using the formula:

$$100 \times \frac{\text{cpm release in test - spontaneous release}}{\text{maximum cpm release - spontaneous release}}$$

Table 14. Clearance of radiolabeled SRBC from mice treated with free or liposome-encapsulated Poly I:C.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		K Value	alpha Value	T/2 (min)	Spleen	Liver	Lung
Control	Mean	.0920	6.55	3.43	137.65	74.02	16.73
	SD	.0204	1.22	.79	40.77	11.22	9.30
Sham Liposome i.v. Day - 2	Mean	.1055	6.98	3.01	157.81	80.50	11.10
	SD	.0254	.68	.72	77.73	11.77	6.35
	p	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Free Poly I:C 50 µg/kg i.v Day -2	Mean	.1296	7.25	2.42	113.81	77.59	6.14
	SD	.0283	0.72	0.58	21.05	9.53	4.75
	p	<0.01	N.S.	<0.05	N.S.	N.S.	<0.05
Free Poly I:C 5 µg/kg i.v. Day -2	Mean	.1110	6.97	2.88	101.44	76.92	12.84
	SD	.0314	0.45	0.70	43.95	5.89	9.04
	p	N.S.	N.S.	N.S.	<0.05	N.S.	N.S.
Free Poly I:C 0.5 µg/kg i.v. Day -2	Mean	.1044	7.25	2.93	144.13	88.68	13.33
	SD	.0149	.33	.39	38.02	9.01	5.56
	p	N.S.	N.S.	N.S.	N.S.	<0.01	N.S.
Poly I:C/ Liposomes 50 µg/kg i.v. Day - 2	Mean	.1581	7.04	1.96	60.97	80.15	4.76
	SD	.0287	.66	.35	28.73	7.29	2.11
	p	<0.001	N.S.	<0.001	<0.01	N.S.	<0.02
Poly I:C/ Liposomes 5 µg/kg i.v. Day - 2	Mean	.1480	7.20	2.07	75.28	77.84	4.17
	SD	.0212	.37	.29	34.25	6.63	2.44
	p	<0.001	N.S.	<0.001	<0.001	N.S.	<0.001
Poly I:C/ Liposomes 0.5 µg/kg i.v. Day - 2	Mean	.1283	8.04	2.50	114.78	85.59	15.18
	SD	.0330	1.28	.71	41.19	24.28	12.99
	p	<0.01	N.S.	<0.01	N.S.	N.S.	N.S.

Table 15. Activation of cytotoxic macrophages by free or liposome-encapsulated Poly I:C.

Treatment	Expt-1		Expt-2		Expt-3	
	CPM	(CI)	CPM	(CI)	CPM	(CI)
Saline Control	58150		8863		242854	
Free Poly I:C	54542	(6)	9331	(-5)	198758	(18)
Sham Liposomes	67722	(-16)	1747	(80)	237407	(2)
Poly I:C/Liposomes	54763	(6)	1217	(86)	44022	(82)

Mice were given poly I:C (50 μg/kg), intraperitoneally, either in free form or encapsulated in liposomes and macrophage cytotoxicity assay was performed two days thereafter. Control mice received the same volume of pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4x10^3$ P388 leukemia cells added to the plate (effector:target cell ratio of 20:1). Cytotoxicity was assayed 48 hours later by pulsing the cells with ^{3}H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 16. Splenic B and T cell numbers in mice treated with free or liposome encapsulated poly I:C.

Treatment Group	Cells/Spleen (x 10-7)	B cells		T cells	
		%	Abs. No. (x 10-7)	%	Abs. No. (x 10-7)
Control	6.73	33	2.20	20	1.30
Free Poly I:C	7.67	41	3.21	25	1.99
Sham Liposomes	5.83	30	1.67	25	1.45
Poly I:C/ Liposomes	7.03	33	2.38	27	1.90

Mice were given free or liposome encapsulated Poly I:C (50 μg/kg), intravenously, two days before assaying for the numbers of splenic B and T cells. The numbers of B and T cells were determined using avidin-conjugated monoclonal anti-sIg or anti-Thy 1 antibodies, respectively, and FITC-labeled avidin. The percentage of B and T cells was determined using a Coulter Epics V flow cytometer and the Immuno data analysis program.

Abs. No. = Absolute numbers of B or T cells

Table 17. Serum interferon levels in squirrel monkeys following administration of various formulations of Poly I:C.

Group	Treatment[1]	Interferon Levels (IU)	
		Expt. 1	Expt. 2
1	Sham Liposomes	<10	<10
2	Free Poly I:C	207	73
3	Poly I:C-LC	95	52
4	Poly I:C/Liposomes	466	506
5	Poly I:C-Dextran	133	238

Five monkeys were injected, intravenously, with the indicated formulations of Poly I:C (400 μg Poly I:C/monkey) and the animals were bled one day after drug administration. Serum interferon levels were determined by the neutral red assay using human foreskin fibroblasts.

[1] Pre-bled samples from two monkeys gave serum interferon levels of <10.

Table 18. Serum interferon levels in mice receiving free or liposome encapsulated AVS-5587 at various times following drug administration.

Treatment	Interferon Titer (IU) Hours Post-Administration		
	4	12	24
Control	<10	10	ND
Sham Liposomes	81	39	<10
Free AVS-5587	28	<10	<10
AVS-5587/Liposomes	25	15	<10

Six week old mice were inoculated, intravenously with free or liposome encapsulated AVS-5587 (10 mg/kg) and bled at the times indicated. Serum interferon levels were determined using mouse L cells and the neutral red assay described in previous quarterly reports.

ND = Not done.

Table 19. Clearance and tissue localization of SRBC following treatment with AVS-5587.

Treatment		Phagocytic Index			RBC/mg Tissue (x1000)		
		$T_{\frac{1}{2}}$ (min)	K Value	alpha Value	Spleen	Liver	Lung
Control	Mean	2.91	0.1078	6.91	129.4	68.1	14.9
	Std. Dev.	0.67	0.031	0.72	24.6	8.2	7.7
AVS-5587 (Free)	Mean	2.51	0.1211	6.78	79.8	74.6	9.3
	Std. Dev.	0.32	0.016	0.42	46.5	6.6	6.2
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Liposomes (Sham)	Mean	2.08	0.1487	7.24	122.0	79.6	7.5
	Std. Dev.	0.44	0.029	0.58	19.2	5.3	3.0
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
AVS-5587 Liposomes	Mean	2.01	0.1515	7.33	101.58	84.17	9.37
	Std. Dev.	0.31	0.020	0.41	8.48	7.04	4.15
	P-value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Mice were injected, intraperitoneally, with AVS-5587 (10 mg/kg), either free or encapsulated in liposomes 2 days before test for clearance of sheep erythrocytes (SRBC) from circulation and its localization in the various organs. Control mice were injected with pyrogen-free saline. $T_{\frac{1}{2}}$ represents the time to clear half the injected SRBC from circulation and K-value represents the rate of clearance. Both values were obtained by plotting the concentration of injected material remaining in circulation at 2, 4, 6, and 10 minutes after injection.

Table 20. Cytotoxic activity of peritoneal macrophages from mice treated with AVS-5587.

Treatment	Statistics	Effector to target ratio		
		40:1	20:1	10:1
Control	Mean CPM	217921	230540	251115
AVS-5587 (Free)	Mean CPM	219377	252971	253195
	C.I.	-1	-10	-1
	P-value	N.S.	N.S.	N.S.
Liposome (Sham)	Mean CPM	234880	232014	237791
	C.I.	-8	-1	-5
	P-value	N.S.	N.S.	N.S.
AVS-5587 Liposomes	Mean CPM	241892	254921	252473
	C.I.	-11	-11	-1
	P-value	N.S.	N.S.	N.S.

Mice were injected intraperitoneally with AVS-5587 (50 mg/kg), either free or encapsulated in liposomes 2 days before harvesting peritoneal cells. Control mice were injected with pyrogen-free saline. Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4x10^3$ P388 leukemia cells added to the plate. Cytotoxicity was assayed by pulsing the cells with ^3H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by the formula:

$$100 \times \frac{\text{CPM in control cultures - CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytotoxicity and cytostasis.

Table 21. Splenic B and T cell numbers in mice treated with AVS-5578, given orally.

Treatment	Cells/Spleen ($\times 10^{-7}$)	B Cells		T Cells	
		%	Abs. # ($\times 10^{-7}$)	%	Abs. # ($\times 10^{-7}$)
Control	2.23	27	0.63	35	0.77
2% $NaHCO_3$	4.87*	33	1.59*	22	1.03
AVS-5587	2.87	29	0.86	21*	0.67

Mice were given AVS-5578 (10 mg/kg) in 2% $NaHCO_3$, by gavage, one day before assaying for the numbers of splenic B and T cells. The numbers of B and T cells were determined using avidin-conjugated monoclonal anti-sIg or anti-Thy-1 antibodies, respectively, and FITC-labeled avidin. The percentage of B and T cells was determined using a Coulter Epics V flow cytometer and the Immuno data analysis program.

Abs. # = Absolute number of B or T cells.

* $P<0.05$.

Table 22. Splenic NK cytotoxicity following intravenous administration of free or liposome encapsulated AVS-5578 on Day -2.

Treatment	% Cytotoxicity at Effector:Target Ratio		
	100:1 ± SD	50:1 ± SD	25:1 ± SD
Control	32 ± 6.7	24 ± 6.5	17 ± 4.9
2% $NaHCO_3$	32 ± 7.1	24 ± 5.0	17 ± 2.7
AVS-5587	42 ± 7.3	33 ± 1.3	25 ± 1.1
Sham Liposomes	38 ± 4.8	30 ± 1.3	20 ± 1.3
AVS-5587/Liposomes	38 ± 1.5	30 ± 1.8	21 ± 1.7

Mice were given free or liposome encapsulated AVS-5587 (10 mg/kg), intravenously, two days prior to assaying for NK cytotoxicity. The drug vehicle was 2% $NaHCO_3$. YAC cells were used as target cells and three mice per treatment group were analyzed. The assay is based on the release of chromium from labeled cells. Percent cytotoxicity was calculated using the formula:

$$100 \times \frac{\text{cpm released in test - spontaneous release}}{\text{maximum cpm released - spontaneous release}}$$

SD = Standard deviation

Table 23. Temperature, differential leukocyte counts and viremia in squirrel monkeys infected with dengue 2.

Monkey		Temp.	Viremia
Controls	Day 0		
802C		102.0	<10
814C		103.0	<10
Infected			
76		100.4	<10
921		101.2	<10
925		103.4	<10
Controls	Day 1		
802C		104.3	<10
814C		103.2	<10
Infected			
76		103.8	>5000
921		102.6	>5000
925		103.8	>5000

Table 23 continued on next page.

Table 23 continued.

Monkey		Temp.	Viremia
Controls	Day 2		
802C		102.6	<10
814C		104.0	<10
Infected			
76		103.4	325
921		104.0	>5000
925		102.2	875
Controls	Day 3		
802C		103.0	<10
814C		102.8	<10
Infected			
76		102.8	125
921		103.0	400
925		102.2	350

Table 23 continued on next page.

Table 23 continued.

Monkey		Temp.	Viremia
Controls	Day 4		
802C		104.0	<10
814C		103.4	<10
Infected			
76		103.6	100
921		103.4	525
925		102.6	120
Controls	Day 5		
802C		103.4	<10
814C		102.6	<10
Infected			
76		101.6	175
921		102.0	75
925[1]		103.4	-

Table 23 continued on next page.

Table 23 continued.

Monkey		Temp.	Viremia
Controls	Day 6		
802C		102.0	<10
814C		101.6	<10
Infected			
76		101.4	75
921		102.2	-
925		103.0	-
Controls	Day 7		
802C		101.0	<10
814C		100.0	<10
Infected			
76		102.2	125
921		101.7	175
925		103.6	-

Table 23 continued on next page.

Table 23 continued.

Monkey		Temp.	Viremia
Controls	Day 8		
802C		100.4	<10
814C		101.8	<10
Infected			
76		101.8	-
921		101.4	-
925		102.6	-

Monkeys were infected subcutaneously with dengue 2 (1 x 10^6 .f.u.; strain 16681) and bled each day following infection. Viremia was measured by plaque titration on mosquito cells. Monkeys were monitored daily for fever using a rectal thermometer.

[1] Monkey 925 developed a nasal discharge five days after infection.

Table 24. Liver enzyme profiles in squirrel monkeys infected with dengue 2.

Monkey		Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls	Day 0						
802C		-	147	189	201	114	-
814C		23	361	187	128	62	0.3
Infected							
76		21	235	383	364	310	0.3
921		82	198	248	250	250	0.4
925		24	120	166	230	132	0.2
Controls	Day 1						
802C		21	192	244	297	127	0.6
814C		26	154	313	160	111	0.4
Infected							
76		17	146	337	313	325	0.4
921		425	132	205	254	236	1.4
925		33	85	193	271	135	0.5

Table 24 continued on next page.

Table 24 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 2						
802C	24	145	199	296	131	0.4
814C	26	95	250	165	107	0.5
Infected						
76	27	338	443	508	359	0.7
921	435	177	241	231	230	1.7
925	33	130	219	258	139	0.6
Controls Day 3						
802C	-	54	166	230	122	0.4
814C	24	85	193	148	118	1.0
Infected						
76	36	385	778	563	342	1.1
921	436	142	238	192	234	3.0
925	35	68	188	212	131	0.7

Table 24 continued on next page.

Table 24 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 4						
802C	8	68	140	192	140	1.6
814C	22	120	158	116	112	1.4
Infected						
76	32	258	576	433	292	1.3
921	381	159	192	183	198	2.7
925	39	88	202	252	137	1.1
Controls Day 5						
802C	19	132	152	195	137	1.6
814C	19	168	304	161	127	1.6
Infected						
76	32	494	561	540	305	1.3
921	344	112	226	180	224	2.2
925[1]	35	116	215	233	139	1.2

Table 24 continued on next page.

Table 24 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 6						
802C	17	77	136	183	146	1.5
814C	18	98	243	156	144	1.3
Infected						
76	37	462	536	611	330	1.8
921	365	344	208	173	197	3.4
925	31	100	163	210	141	1.0
Controls Day 7						
802C	-	54	120	156	141	-
814C	18	59	173	142	164	0.7
Infected						
76	29	182	326	396	319	0.9
921	-	72	141	138	180	0.6
925	28	99	133	204	144	0.7

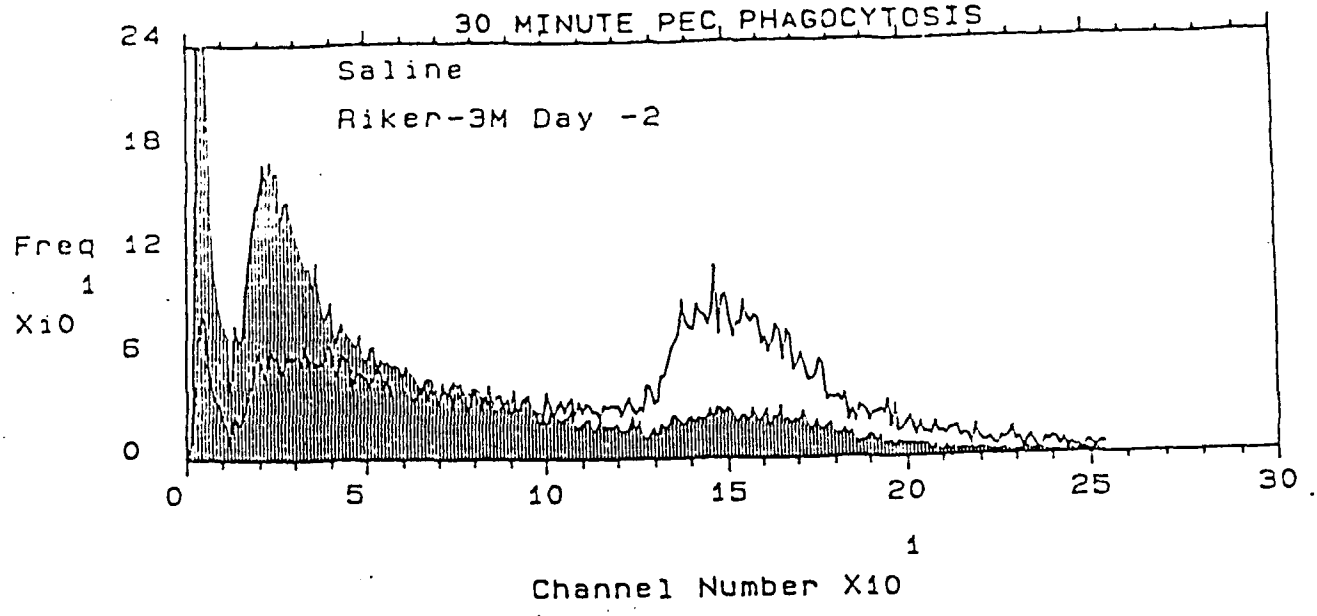
Table 24 continued on next page.

Table 24 continued.

Monkey	Gamma GTP IU/ml	LDH IU/ml	SGOT IU/ml	SGPT IU/ml	Alk. Phos. IU/ml	Total Bil. mg/dl
Controls Day 8						
802C	15	57	141	172	160	1.3
814C	19	148	165	136	191	0.6
Infected						
76	33	221	356	386	418	1.1
921	313	201	178	164	300	4.2
925	28	171	142	242	181	1.5

Monkeys were infected subcutaneously with dengue 2 (1×10^6 .f.u.; strain 16681) and bled each day following infection. Viremia was measured by plaque titration on mosquito cells. Monkeys were monitored daily for fever using a rectal thermometer.

[1] Monkey 925 developed a nasal discharge five days after infection.



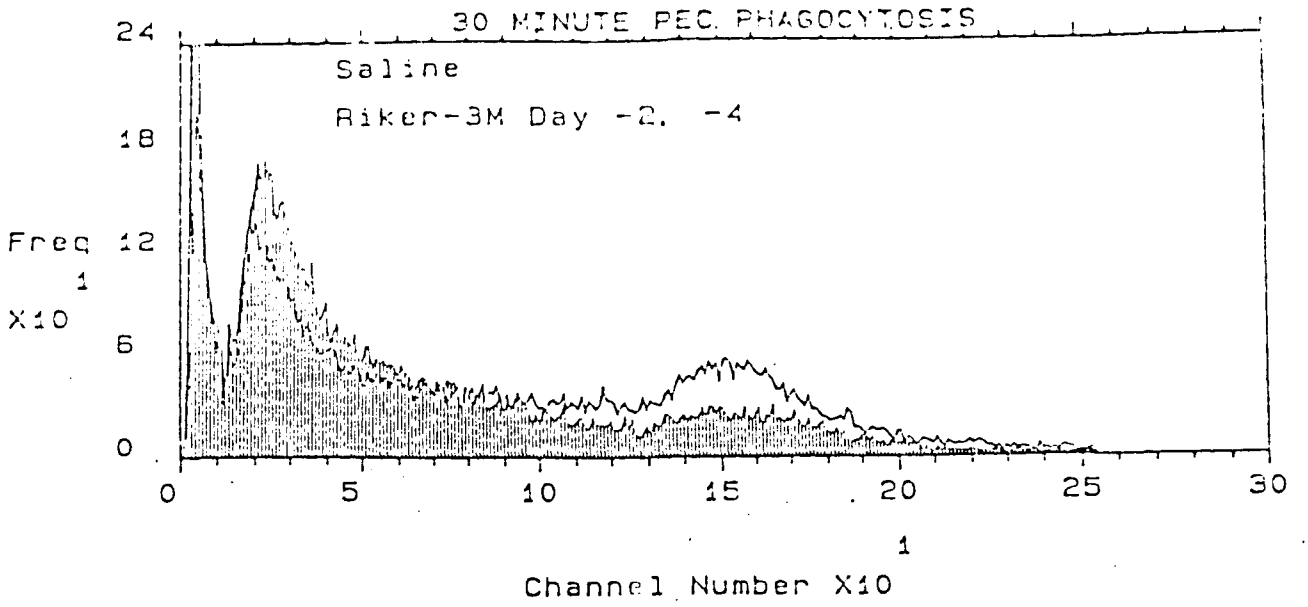
% Phagocytic Cells

Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2)	70*	9	44*	18

Figure 1. Phagocytosis by peritoneal exudate cells following oral administration of Riker-3M compound on day -2.

Mice were given Riker-3M compound (10 mg/kg), orally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

* $p < 0.01$

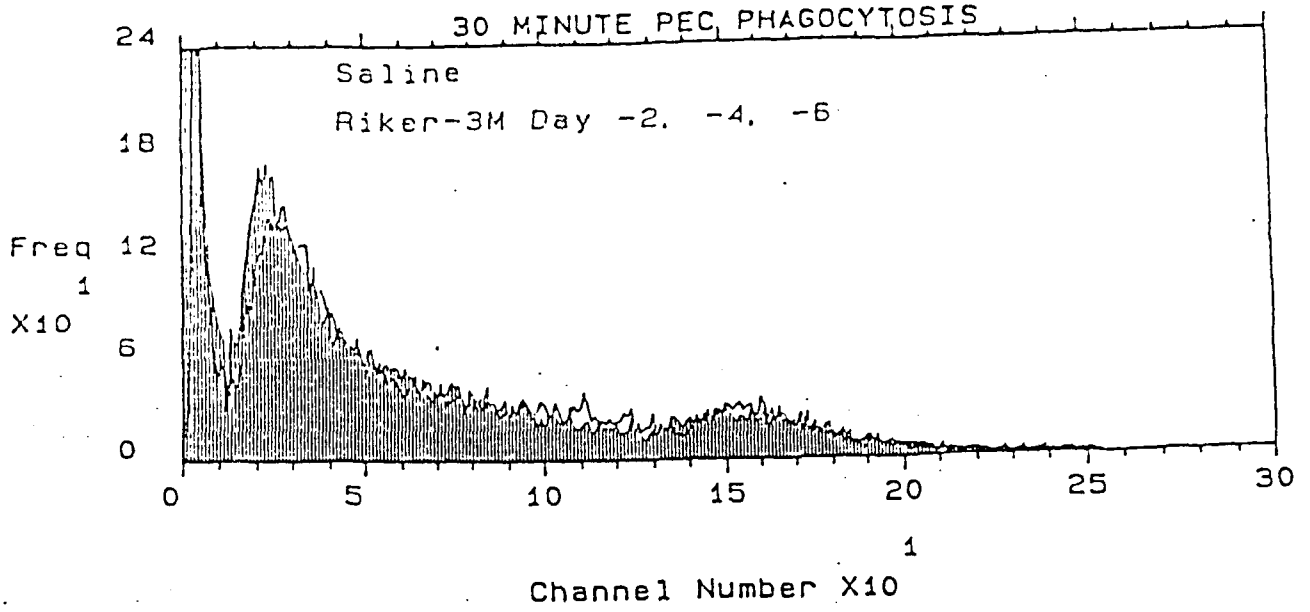


Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4)	53	9	33*	11

Figure 2. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M compound on days -2 and -4.

Mice were given Riker-3M compound (10 mg/kg), intraperitoneally, two and four days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

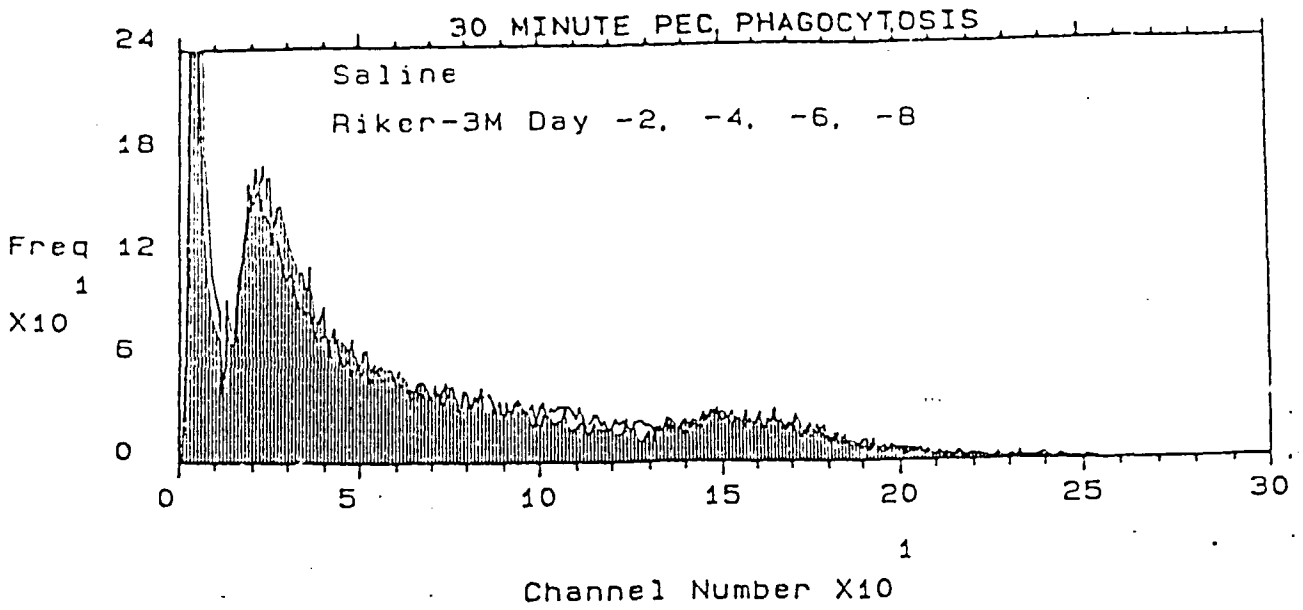
* $p < 0.05$



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4,-6)	37	9	22	6

Figure 3. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M compound on days -2, -4 and -6.

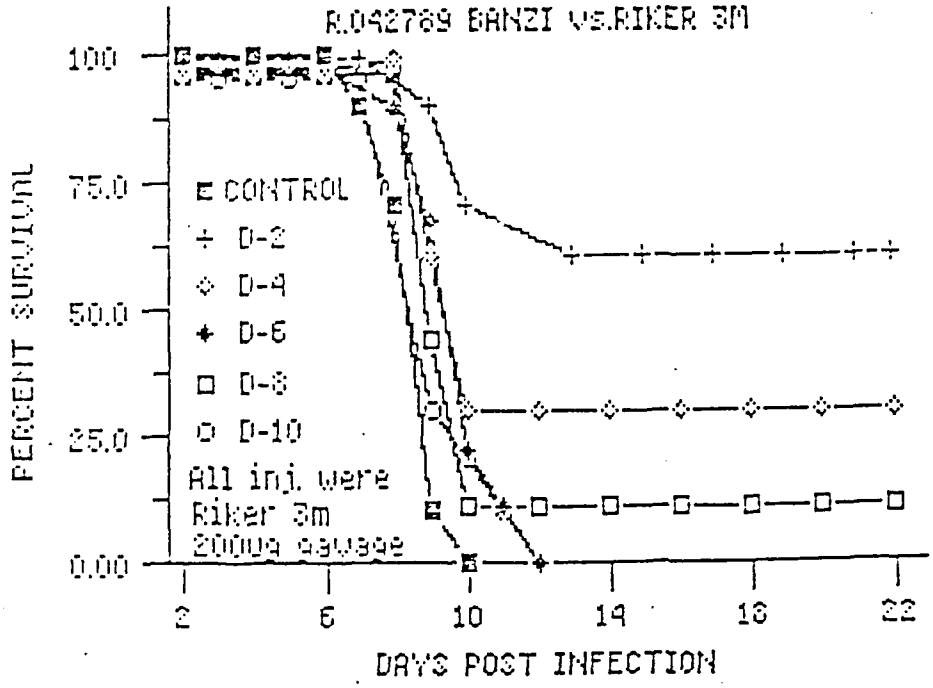
Mice were given Riker-3M compound (10 mg/kg), intraperitoneally, two, four and six days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



	% Phagocytic Cells			
Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
1% Lactic Acid	37	10	20	7
Riker-3M in 1% Lactic Acid (Day -2,-4,-6,-8)	46	10	25	12

Figure 4. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of Riker-3M compound on days -2, -4, -6 and -8.

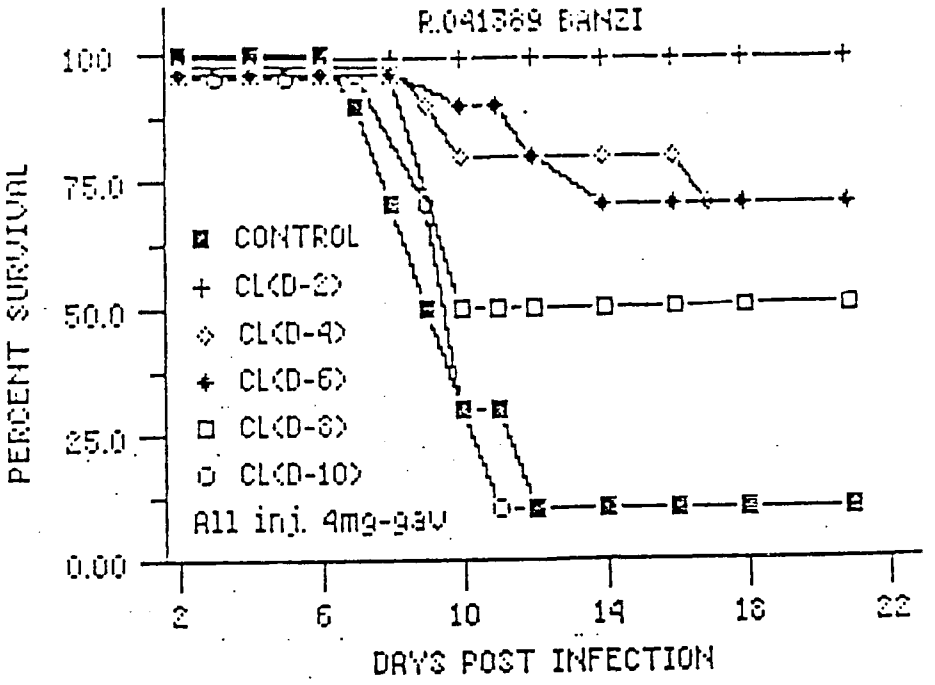
Mice were given Riker-3M compound (10 mg/kg), intraperitoneally, two, four, six and eight days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	Geometric Mean Survival Time (days)	P-value
Control- 1% lactic acid	8.7	—
Riker-3M, 10 mg/kg prior to infection		
day-2	15.9	<.005
day-4	12.0	<.05
day-6	9.8	NS
day-8	10.2	NS
day-10	9.6	NS

Figure 5. Prophylactic activity of Riker-3M compound in Banzi encephalitis.

Mice received by gavage 10 mg/kg of Riker-3M compound in a 1% lactic acid carrier on the day indicated. Mice were challenged intraperitoneally with 1 LD_{80} of virus on day 0 and examined daily for 21 days. Ten mice were included in each group. Significance levels were determined using Wilcoxon rank analysis.



Treatment	Geometric Mean Survival Time (days)	P-value
Saline Control	10.2	—
CL 246738 200 mg/kg prior to infection		
day-2	21.0	<.001
day-4	17.5	<.01
day-6	17.7	<.01
day-8	14.5	<.05
day-10	10.6	NS

Figure 6. Prophylactic activity of CL 246738 in Banzi encephalitis.

Mice received by gavage 200 mg/kg of CL 246738 on the day indicated. Mice were challenged intraperitoneally with 1 LD_{80} of virus on day 0 and examined daily for 21 days. Ten mice were included in each group. Significance levels were determined using Wilcoxon rank analysis.

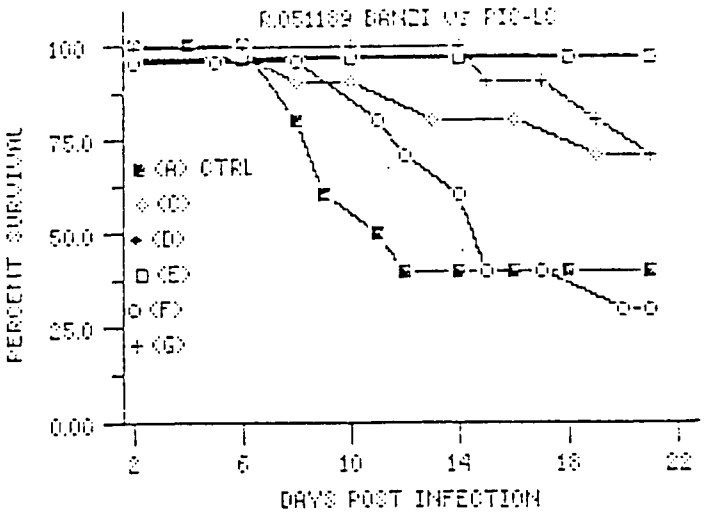


Figure 7a

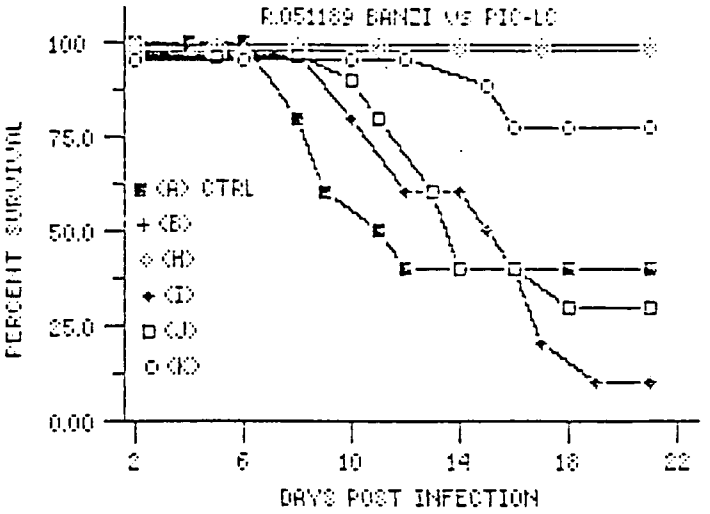
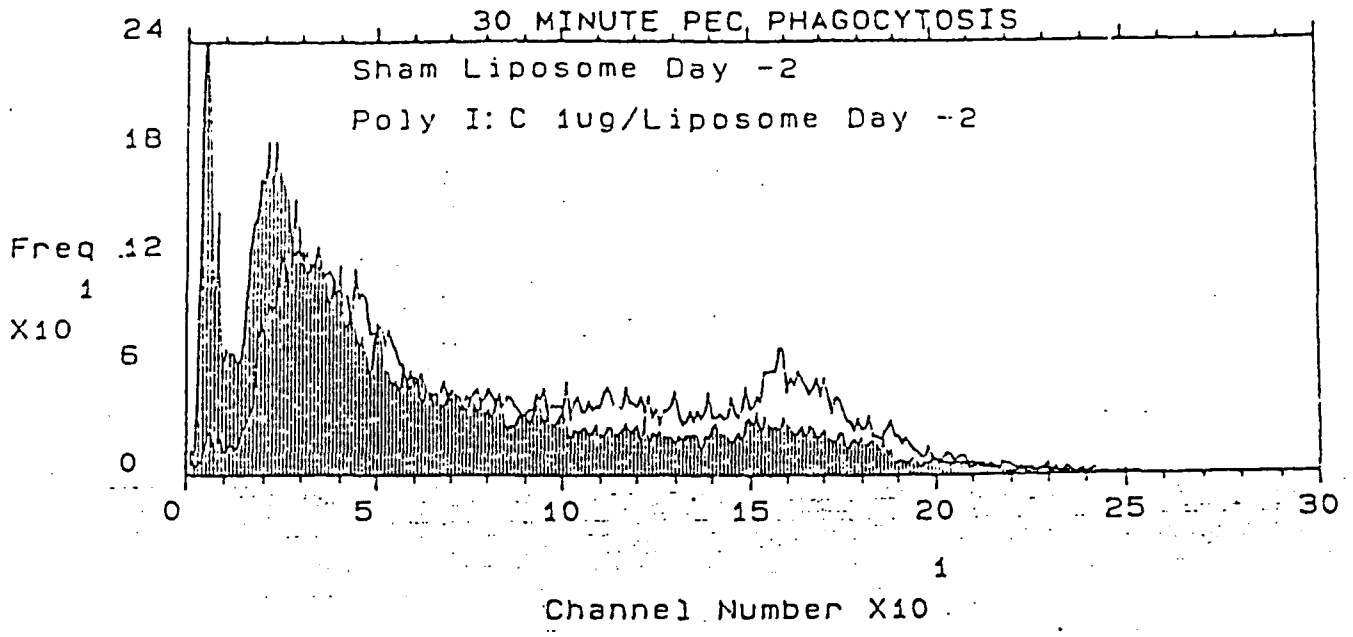


Figure 7b

Group	Day of treatment
A.	-10, -5, 0, +5, +10 (saline control)
C.	-10, -5, 0, +5, +10
D.	-11, -6, -1,+4, +9
E.	-12, -7, -2,+3, +8
F.	-13, -8, -3, +2, +7
G.	-14, -9, -4, +1, +6

Group	Day of treatment
A.	-20, -15, -10, -5, 0, +5, +10 (saline control)
B.	-20, -15, -10, -5, 0, +5, +10
H.	-21, -16, -11, -6, -1, +4, +9
I.	-22, -17, -12, -7, -2, +3, +8
J.	-23, -18, -13, -8, -3, +2, +7
K.	-24, -19, -14, -9, -4, +1, +6

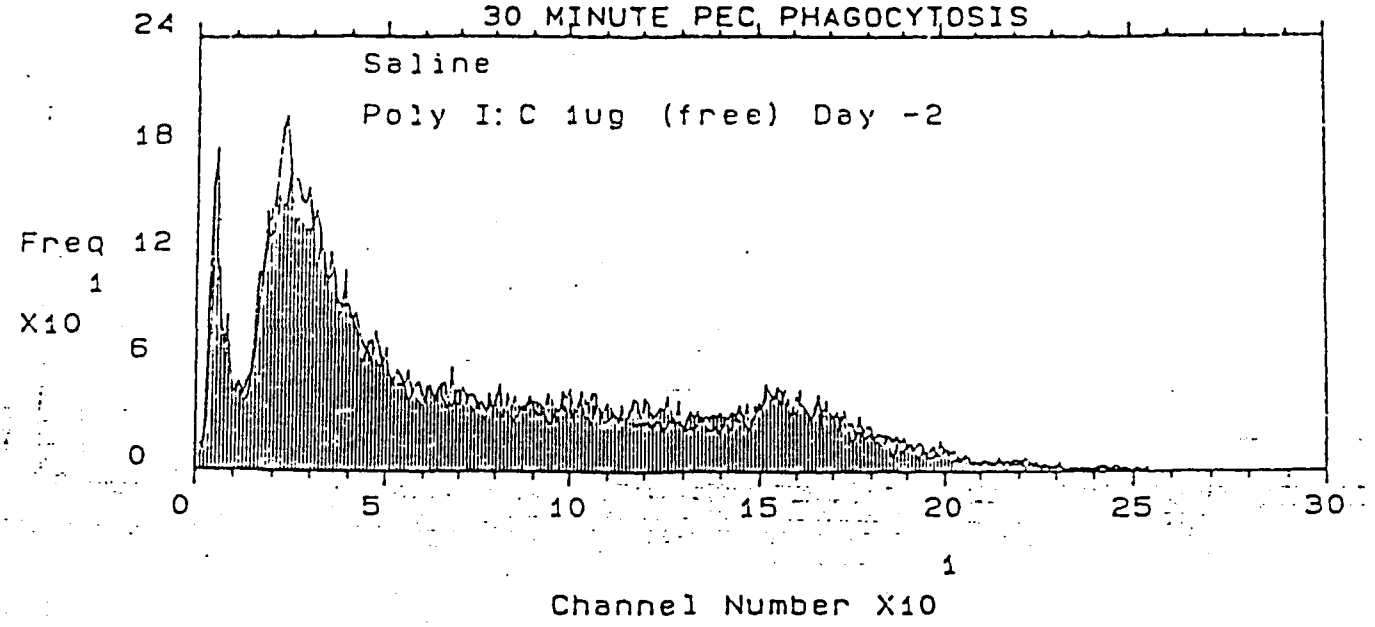
Figure 7. Resistance to Banzi virus following five (Figure 7a) or seven (Figure 7b) i.v. injections with Poly I:C-LC.



	% Phagocytic Cells			
Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Sham Liposomes	47	10	22	6
Poly I:C/Liposomes	56	11	36	10

Figure 8. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

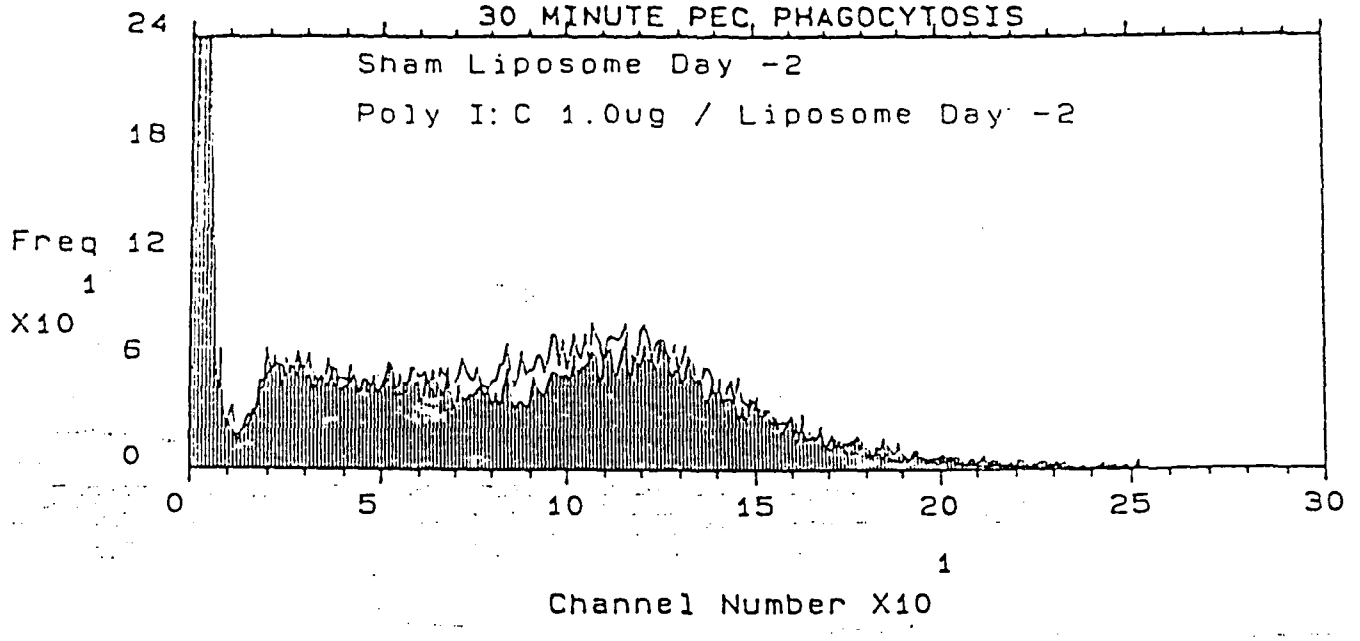
Mice were given liposome encapsulated Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



Treatment	% Phagocytic Cells			
	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Control	47	10	30	7
Free Poly I:C	45	10	27	9

Figure 9. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of free Poly I:C on day-2.

Mice were given free Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

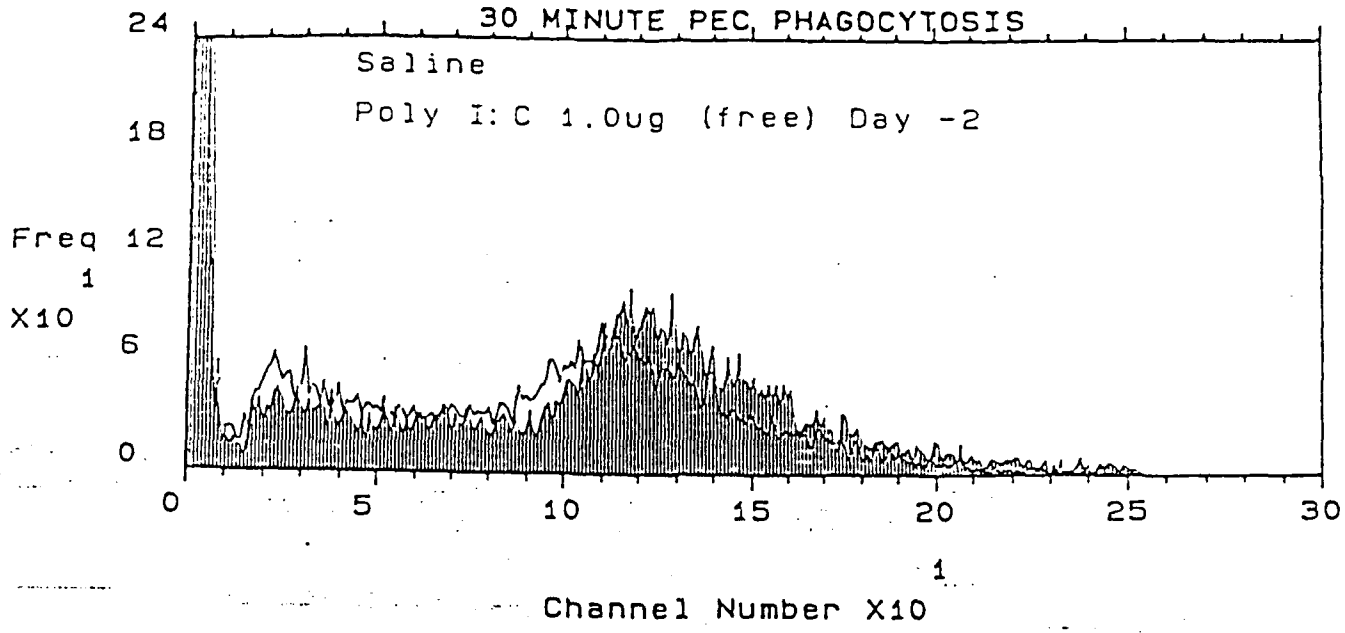


% Phagocytic Cells

Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Sham Liposomes	54	11	38	6
Poly I:C/Liposomes	60	13	44	3

Figure 10. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated Poly I:C on day -2.

Mice were given liposome encapsulated Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.



% Phagocytic Cells

Treatment	Total	Log 1	Log 2	Log 3
Channel No.	61-255	61-85	86-171	172-255
Control	70	10	51	8
Free Poly I:C	51	8	39	5

Figure 11. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of free Poly I:C on day-2.

Mice were given free Poly I:C (50 μg/kg), intraperitoneally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis in the figure represents log fluorescence intensity plotted on a three cycle scale and the y axis represents number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integrating the areas under the curve. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the control.

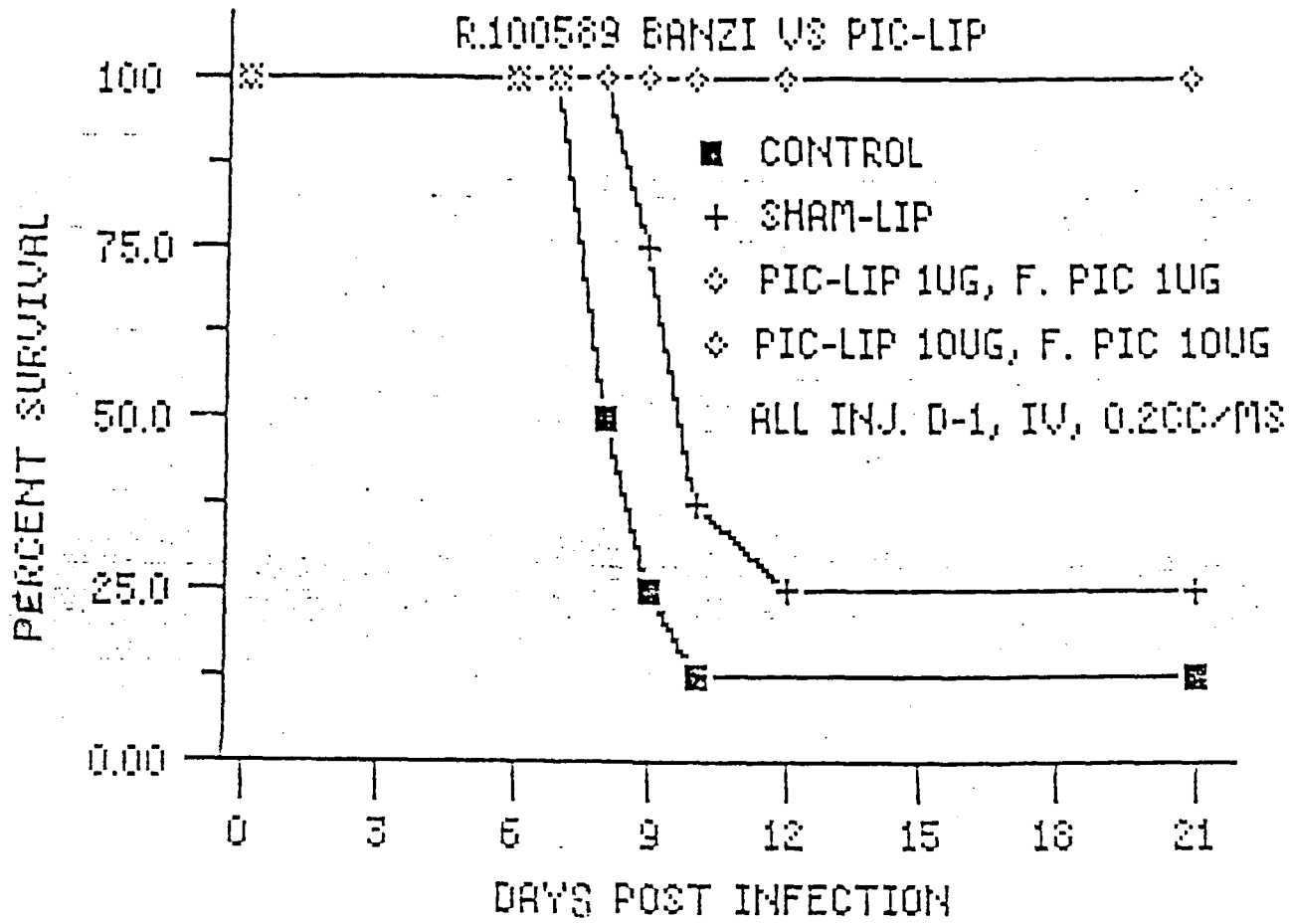


Figure 12. Augmentation of resistance to Banzi virus by liposomal or free Poly I:C administered one day prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated poly I:C (50 or 500 μg/kg) one day prior to challenge with 1 LD80 of Banzi virus. Note that <> represents both free and liposomal drug at 50 and 500 μg/kg.

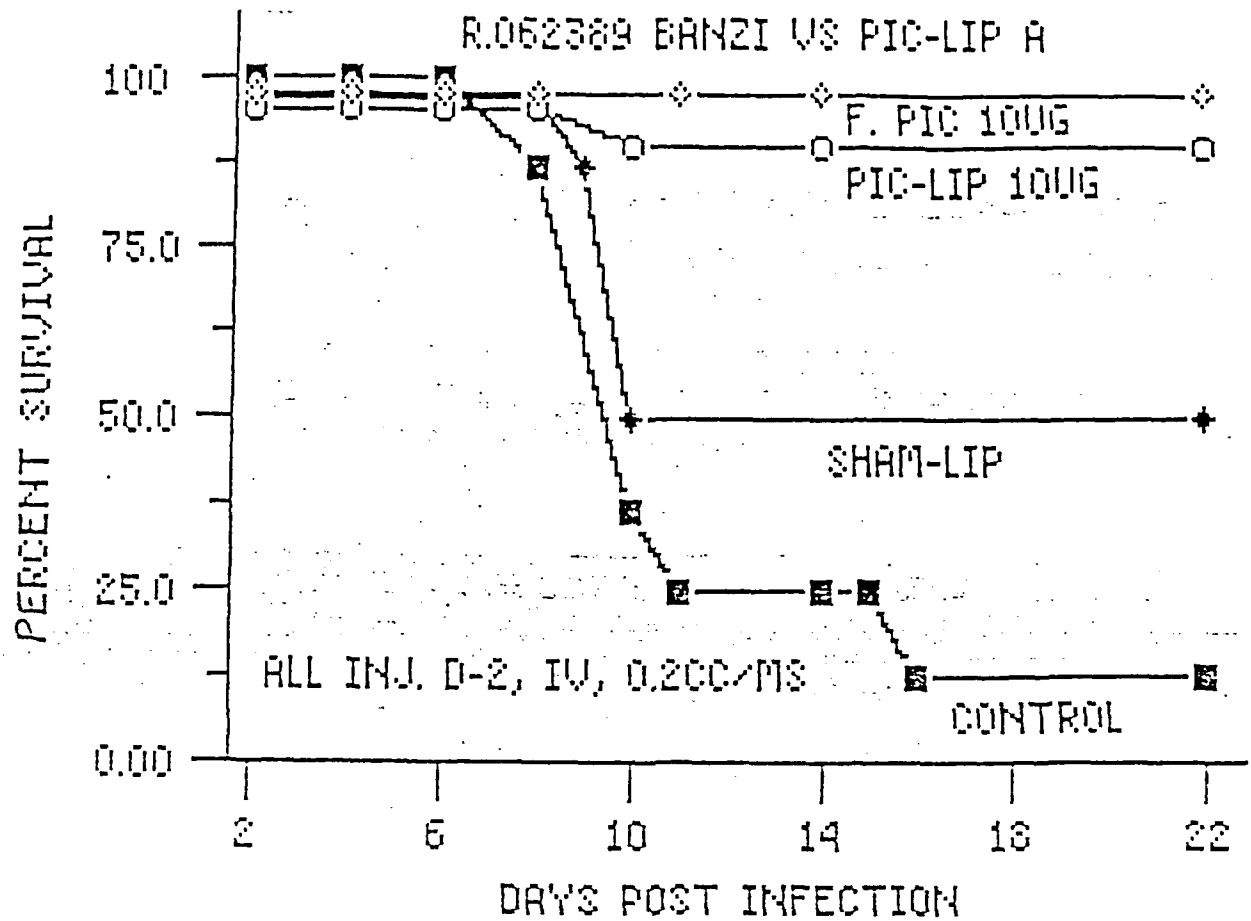


Figure 13. Augmentation of resistance to Banzi virus by liposomal or free Poly I:C administered two days prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated poly I:C (500 μg/kg) two days prior to challenge with 1 LD80 of Banzi virus.

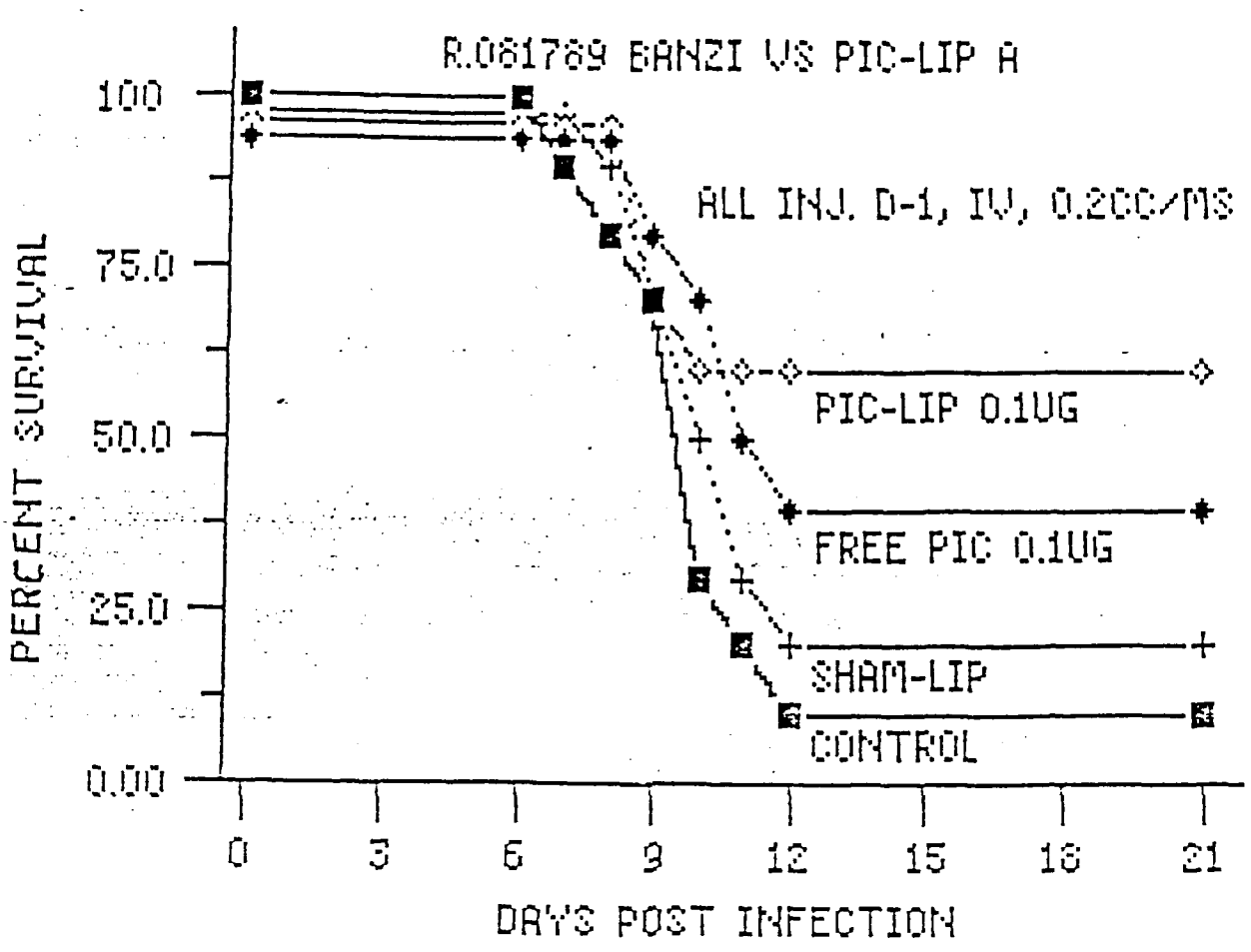


Figure 14. Augmentation of resistance to Banzi virus by low dose liposomal or free poly I:C administered one day prior to infection.

Six-week-old C3H/Hen mice were intravenously inoculated with either free or liposome-encapsulated poly I:C (5 μg/kg) one day prior to challenge with 1 LD80 of Banzi virus.

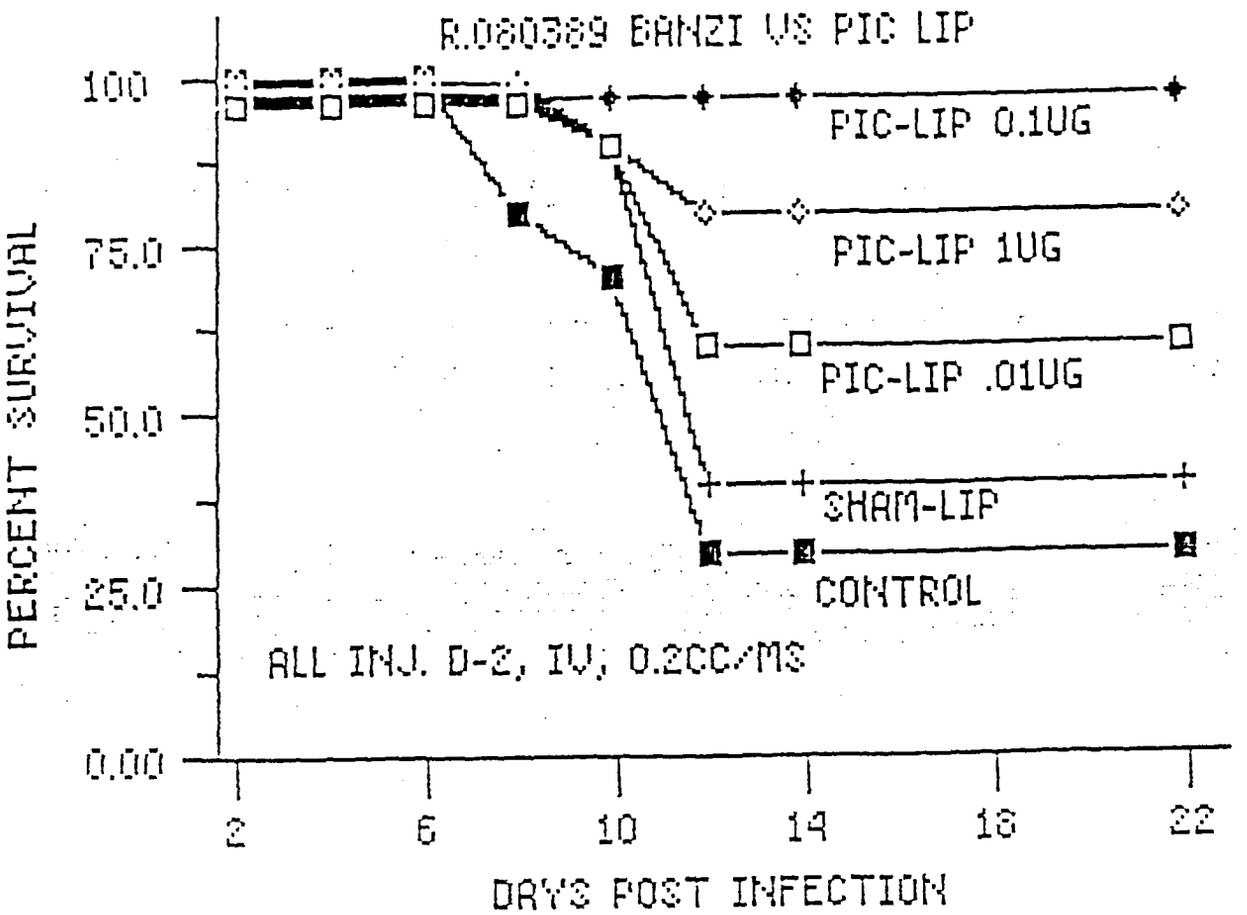
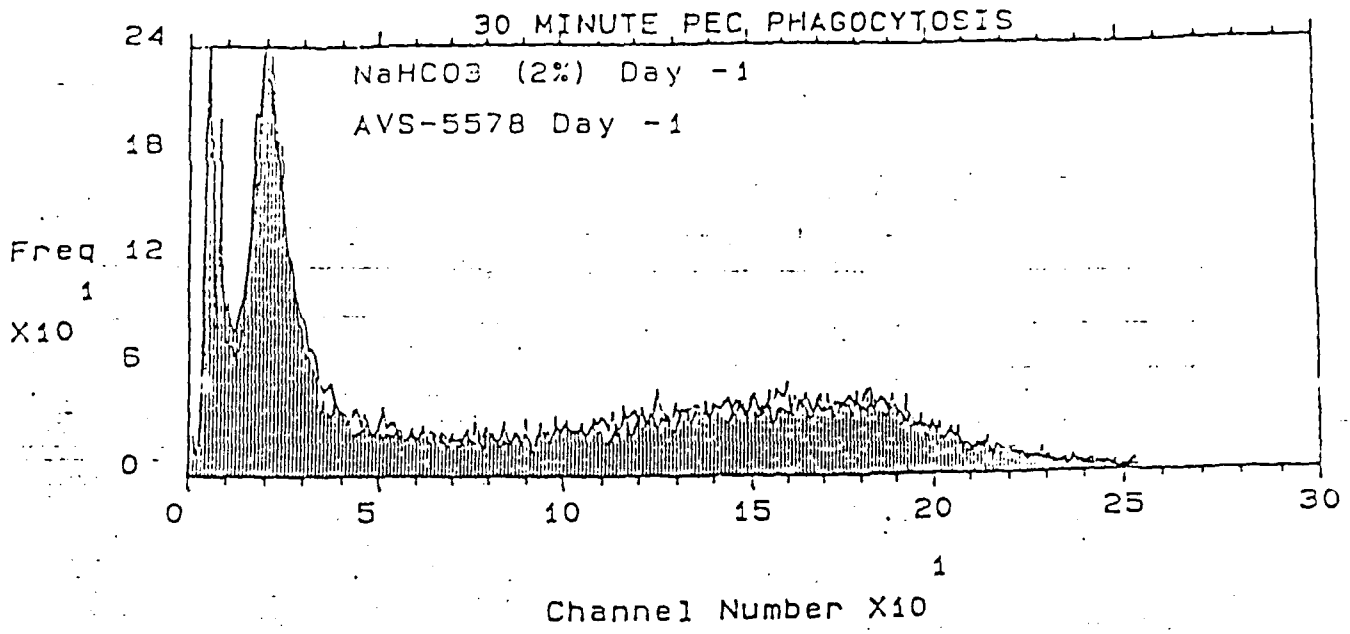


Figure 15. Augmentation of resistance to Banzi virus by low dose liposomal Poly I:C administered two days prior to infection.

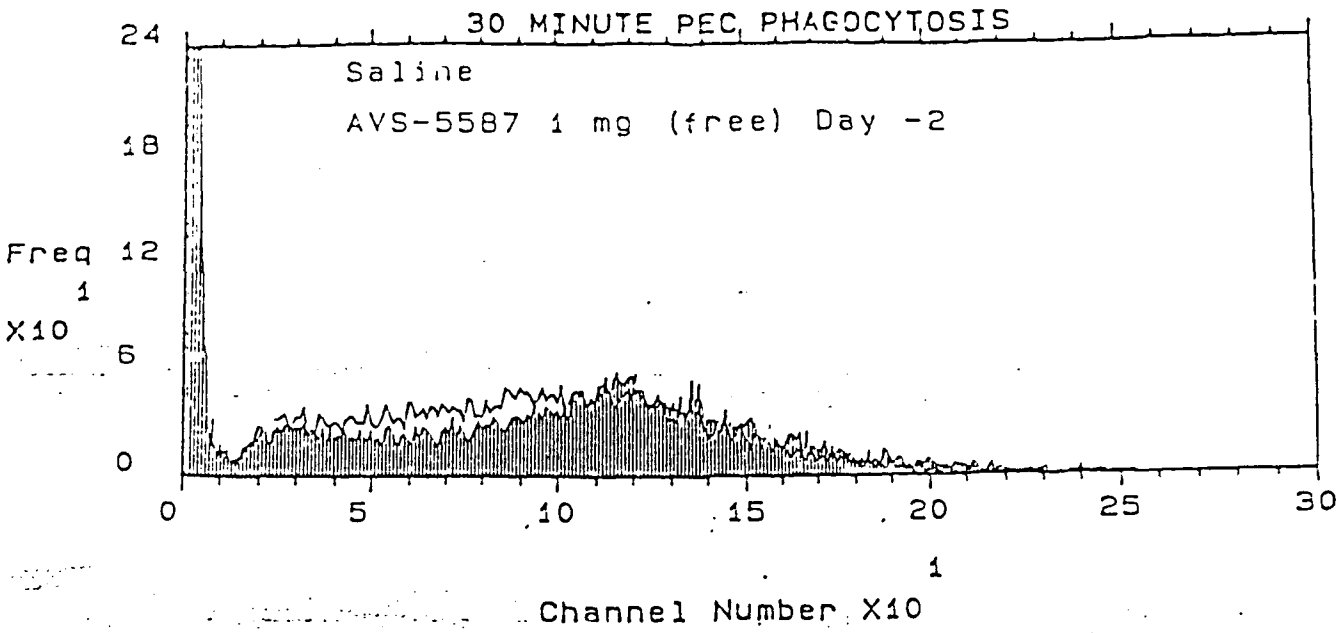
Six-week-old C3H/Hen mice were intravenously inoculated with liposome- encapsulated poly I:C (50, 5, or 0.5 μg/kg) two days prior to challenge with 1 LD80 of Banzi virus.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
2% $NaHCO_3$		49	5	26	18
AVS-5587		51	5	29	17

Figure 16. Phagocytosis by peritoneal exudate cells following oral administration of AVS-5587 on day -1.

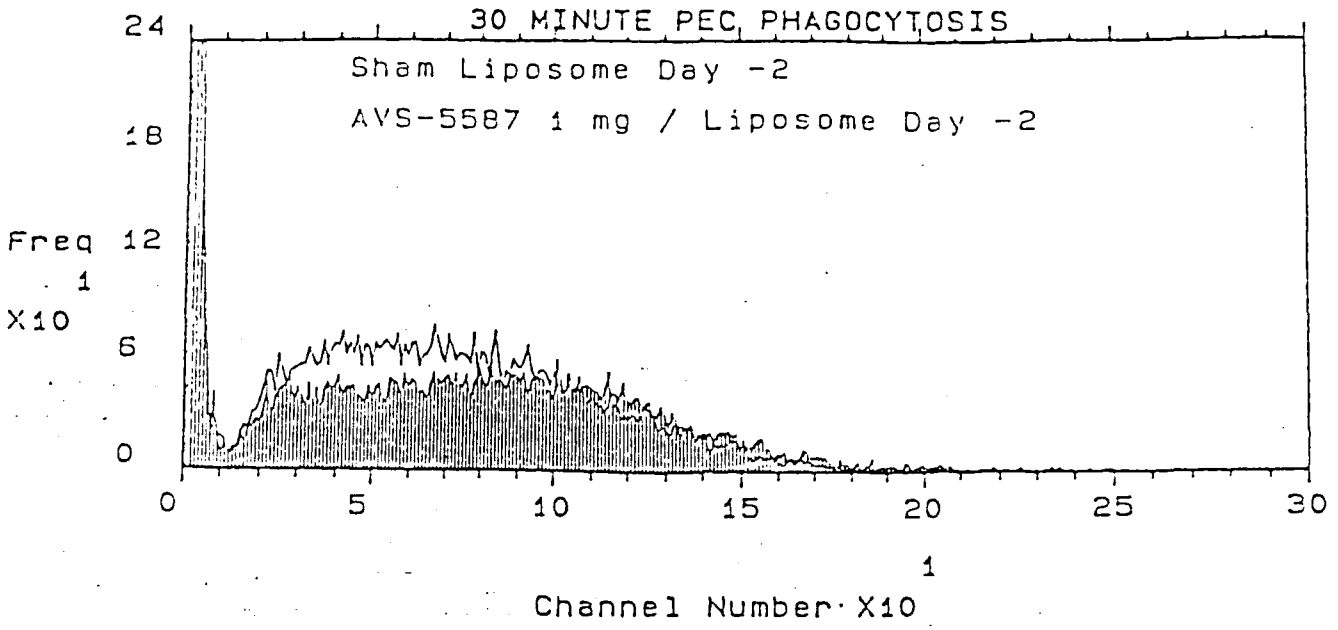
Mice were given AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, orally, one day prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
$NaHCO_3$		43	7	30	6
AVS-5587		41	9	29	3

Figure 17. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of AVS-5587 on day -2.

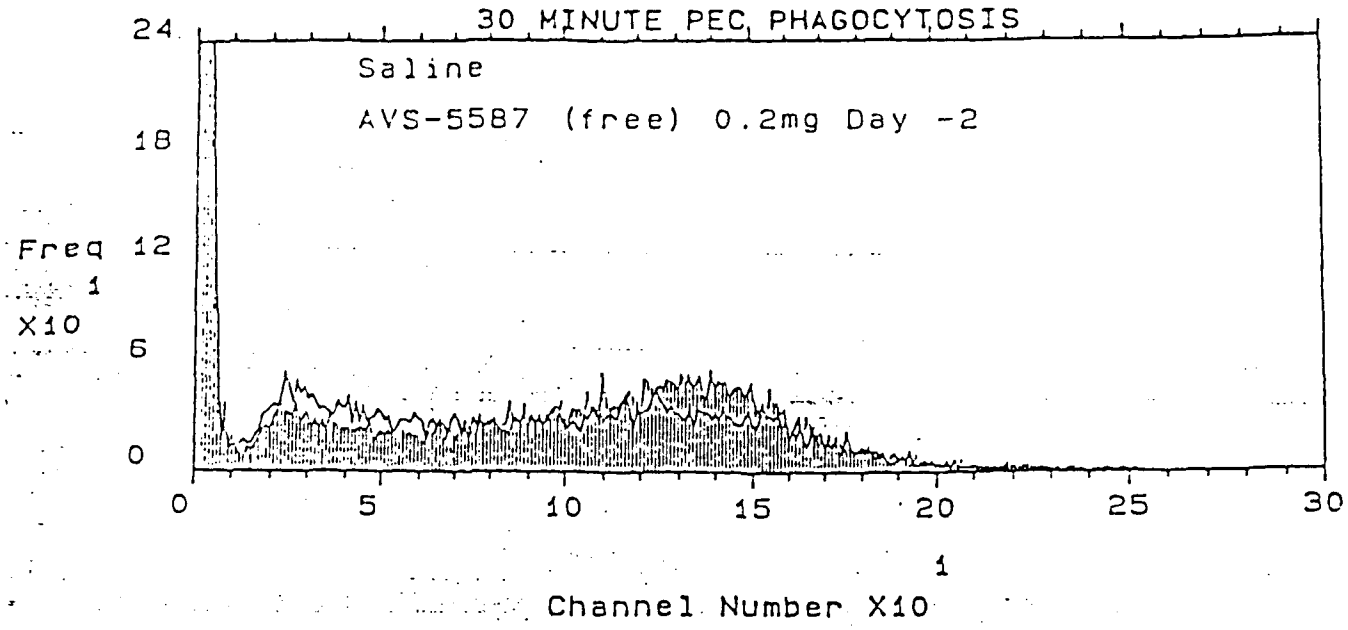
Mice were given AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		42	12	27	2
AVS-5587/Liposomes		43	17	25	1

Figure 18. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated AVS-5587 on day -2.

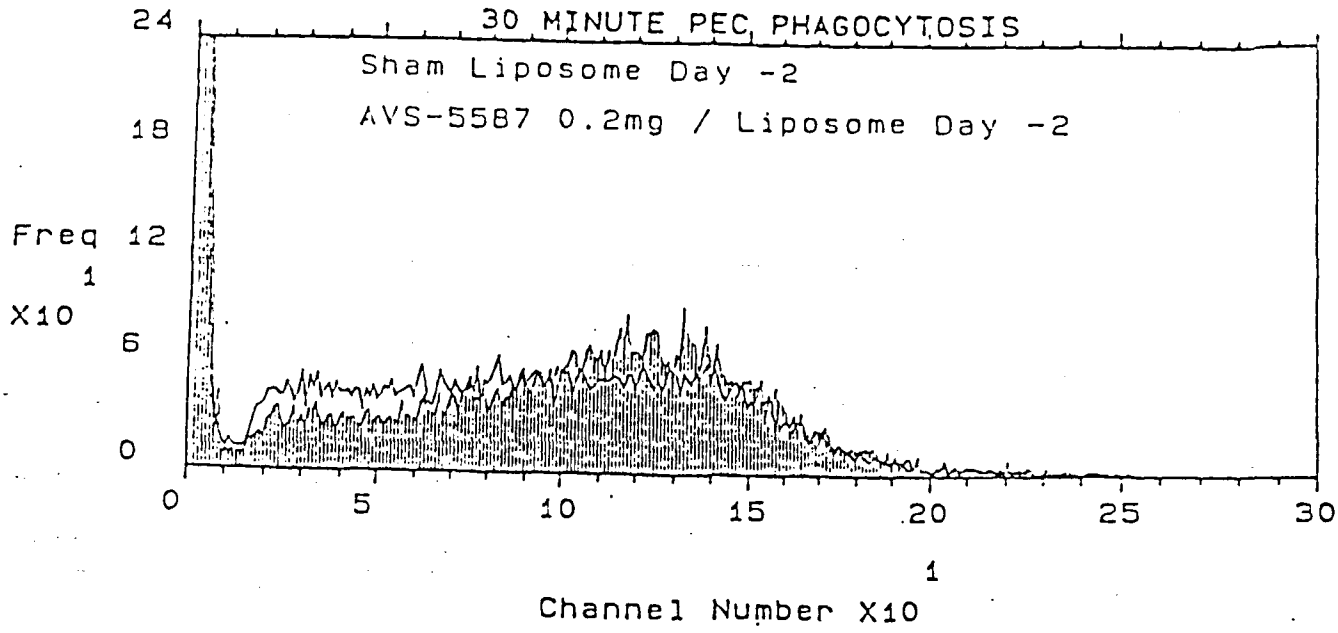
Mice were given liposome encapsulated AVS-5587 (10 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the sham liposome control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
$NaHCO_3$		44	6	33	5
AVS-5587		37	7	26	4

Figure 19. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of AVS-5587 on day -2.

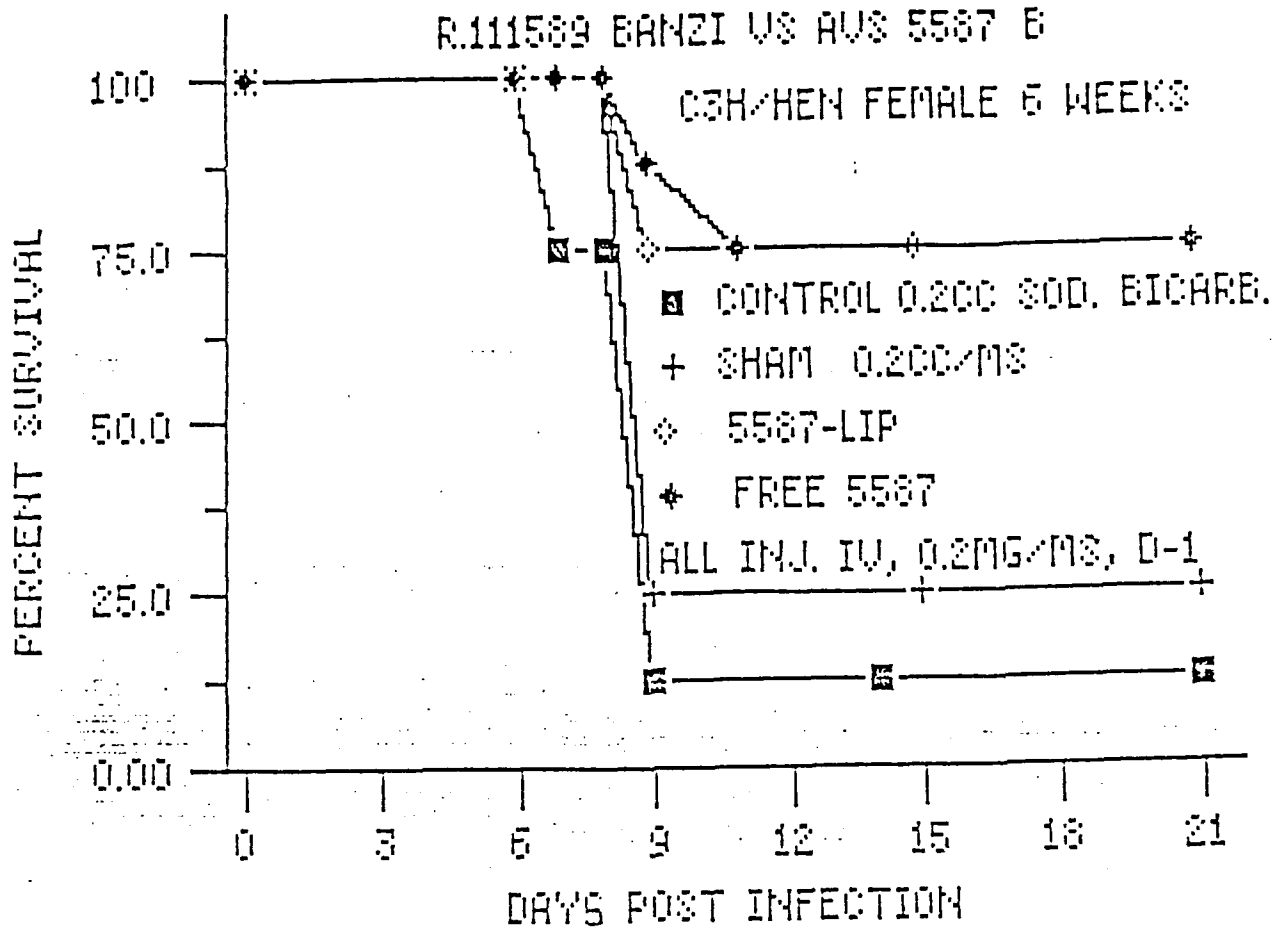
Mice were given AVS-5587 (50 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the $NaHCO_3$ control.



Treatment	Channel No.	% Phagocytic Cells			
		Total 61-255	Log 1 61-85	Log 2 86-171	Log 3 172-255
Sham Liposomes		64	10	48	6
AVS-5587/Liposomes		57	13	41	4

Figure 20. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of liposome encapsulated AVS-5587 on day -2.

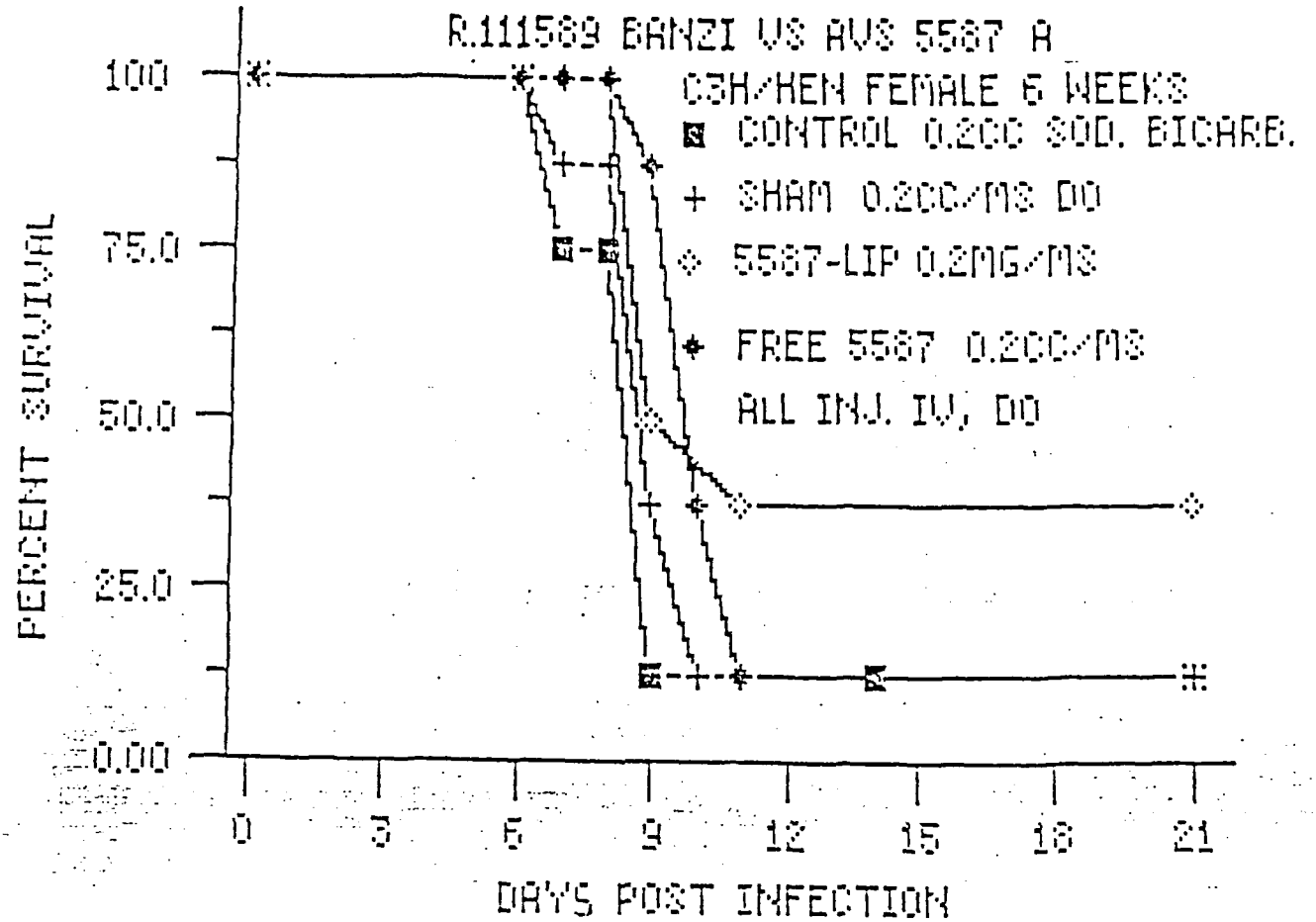
Mice were given liposome encapsulated AVS-5587 (50 mg/kg) in 2% $NaHCO_3$, intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated *S. aureus* by flow cytometry. The x axis represents log fluorescence intensity plotted on a three cycle scale and the y axis represents the number of cells. The total number of phagocytic cells and the percentages in logs 1, 2 and 3 were determined by integration. The fluorescence in channels 1-60 represents primarily autofluorescence. The shaded curve is the data for the sham liposome control.



Treatment	MST (Days)	P Value
Control	9.40	-
Sham Liposomes	11.12	NS
AVS-5587/Liposomes	16.99	<0.05
Free AVS-5587	17.22	<0.05

Figure 21. Augmentation of resistance to Banzi virus by liposomal or free AVS-5587 administered one day prior to infection.

Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated AVS-5587 (10 mg/kg) one day prior to challenge with 1 LD_{80} of Banzi virus.



Treatment	MST (Days)	P Value
Control	9.40	-
Sham Liposomes	9.95	NS
AVS-5587/Liposomes	12.68	NS
Free AVS-5587	10.34	NS

Figure 22. Augmentation of resistance to Banzi virus by liposomal or free AVS-5587 administered on the day of virus infection.

Six week old C_3H/HeN mice were intravenously inoculated with either free or liposome-encapsulated AVS-5587 (10 mg/kg) on the day of challenge with 1 LD_{80} of Banzi virus.